AMINO ACID AND / OR MULTIPLE MICRONUTRIENT SUPPLEMENTATION IN ENVIRONMENTAL ENTEROPATHY: EFFECTS ON SMALL INTESTINAL BARRIER STRUCTURE AND FUNCTION

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For Grace, Carmelita and Dominic.

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STATEMENT OF ORIGINALITY

I, John Richard Louis-Auguste, confirm that the research included within this thesis is my own work or that where it has been carried out in collaboration with, or supported by others, that this is duly acknowledged below and my contribution indicated. Previously published material is also acknowledged below.

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DETAILS OF COLLABORATIONS

- Identification of potential trial participants, monthly and *ad hoc* reviews, and compliance monitoring were conducted by Sr Rose Banda, Mr John Mbewe and the late Mr Coillard Kaunga based at the Misisi research clinic.
- Participant screening, consenting, and enrolment was undertaken with the help of Sr Rose Banda, based at the Misisi research clinic.
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ABSTRACT

Environmental enteropathy (EE) is thought to underlie stunting, a widely prevalent phenomenon in the developing world with lifelong detrimental consequences. EE is characterised by increased intestinal permeability and reduced absorptive capacity in a chronically inflamed gut, and occurs in nutritionally vulnerable populations.

Deficits of amino acids and micronutrients may cause intestinal immune and barrier dysfunction, mediated through cellular nutrient sensing pathways. Mechanistic target of rapamycin complex 1 (MTORC1) is the most important of these, and is of particular interest in EE due to its roles in both epithelial and immune cell function.

It was hypothesised that supplementation with the amino acids (AA) L-Glutamine, L-Tryptophan and L-Leucine +/- high dose multiple micronutrients (MM) could modify the abnormal small intestinal histology and permeability seen in EE, and would modify lamina propria lymphocyte (LPL) MTORC1 signalling. A randomised controlled trial was therefore undertaken. Assessments of many of the patho-physiological domains of EE (absorptive area; mucosal inflammation; microbial translocation; permeability; systemic immune activation; host-microbiota interactions; enterohumoral signalling), as well as LPL MTORC1 signalling, were performed in 84 healthy adult Zambians with EE before and after 16 weeks' supplementation.

AA supplementation improved villus height, whereas AA with MM supplementation improved *in vivo* barrier permeability. Supplementation did not affect LPL MTORC1 signalling; however, MTORC1 activity correlated with villus height and the size of the LP T_{H1} population. Metabonomics identified several potentially relevant metabolites which were upregulated by the interventions. Of note, other observed abnormalities (e.g. elevated translocation/activation markers; depressed levels of GLP2) did not change with supplementation and were mainly not correlated with the improvements in histology and permeability, suggesting that one or more pathophysiological domains (e.g. absorptive area; permeability) may be independently modifiable.

These results and others explored in this thesis provide new insights into the complex relationship between nutrition, intestinal health, and EE.

TABLE OF CONTENTS

Front matter **1**

Abstract 7

Table of contents 8

List of figures 11

List of tables 13

List of abbreviations 15

Chapter 1. Introduction **16**

1.1 Stunting and its consequences in the developing world 16

1.2 Causes of stunting: undernutrition, infection, WASH, and environmental enteropathy 19

1.3 Environmental enteropathy (EE) 23

1.4 Role of nutrition in environmental enteropathy 44

1.5 Other interventions for environmental enteropathy 48

1.6 The mechanistic target of rapamycin complex 1 (MTORC1) cellular nutrient sensing pathway and its therapeutic potential in EE 50

1.7 Summary 69

1.8 Hypothesis, aims, and scope of this thesis 71

Chapter 2. Amino acids and / or multiple micronutrients in adult Zambians with environmental enteropathy (the AMAZE study): trial overview **75**

2.1 Introduction 75

2.2 Study setting and participants 75

2.3 Recruitment and informed consent procedures 77

2.4 Study flowchart and schedule of assessments 79

2.5 Randomisation and blinding procedures 81

2.6 Study intervention 81

2.7 Endpoints 87

2.8 Statistical considerations 88

2.9 Data collection 90

2.10 Regulatory aspects 91

2.11 Specific ethical considerations 92

Chapter 3. Methods **96**

3.1 Overview of available techniques for studying the intestinal barrier in environmental enteropathy 96

3.2 Morphometric analysis of endoscopic small intestinal biopsies 99

3.3 Confocal laser endomicroscopic assessment of small intestinal dysfunction in EE 102

3.4 Assessing lamina propria T cell MTORC1 activity by flow cytometry 112

3.5 Identification and quantification of lamina propria T_{H1} cells 125

3.6 Cell viability and cell permeabilisation assay 128

3.7 Urinary metabonomics 129

3.8 Quantification of plasma CRP, sCD14 & GLP2 by ELISA 131

3.9 *Limulus* amoebocyte assay for the detection of lipopolysaccharide 133

3.10 Assessment of nutritional status and body composition 134

Chapter 4. Results: AMAZE trial overview, primary outcomes, and safety **138**

4.1 Study participants 138

4.2 Effect of AA or MM supplementation on proximal small intestinal absorptive area 142

4.3 Effect of amino acid or multiple micronutrient supplementation on small intestinal barrier dysfunction as assessed by CLE 146

4.4 Effect of AA or MM supplementation on mucosal T lymphocyte MTORC1 activity 148

4.5 Effect of HIV status 152

4.6 Correlation between small intestinal structure, function and lamina propria lymphocyte nutrient responsiveness 155

4.7 Safety and adverse events 156

Chapter 5. Results: secondary outcomes **158**

5.1 Lamina propria T_{H1} cells and the role of MTORC1 signalling 158

5.2 Correlation between T cell type and activation status on MTORC1 activity 159

5.3 Impaired MTORC1 signalling response to *in vitro* nutrient stimulation in a subset of lamina propria $CD3^+$ lymphocytes 161

5.4 Effect of HIV status on lamina propria $CD4^+$ T lymphocyte population 162

5.5 Characteristics of the lamina propria immune compartment in adults with EE 163

5.6 Serum markers of intestinal barrier function 164

5.7 Metabonomics 167

5.8 Enterohumoral trophic signalling assessed by plasma GLP2 levels 173

5.9 Global nutritional status and body composition 174

Chapter 6: Discussion **176**

6.1 Overview 176

6.2 Summary of findings 177

6.3 Enteropathy characteristics in the study population 179

6.4 Impact of supplementation on the intestinal barrier in EE 183

6.5 Potential role of MTORC1 signalling 186

6.6 Effect of HIV on the mucosal immune compartment 189

6.7 Insights into host-microbial interactions 191

6.8 Enterohumoral signalling in EE: role of GLP2 193

6.9 Anthropometry 194

6.10 Study strengths 195

6.11 Study weaknesses 196

6.12 Methodological comments 198

6.13 Conclusions and future directions 201

Appendices **204**

1 Patient information sheet (English version) 205

2 Consent form 209

3 Case reporting forms 210

4 ImageJ morphometry macro 219

5 Incubation media for MTORC1 flow cytometry assay 221

References **222**

LIST OF FIGURES

Chapter 1.

1.1. Global prevalence of stunting in children under the age of 5 16
1.2. Long term and intergenerational consequences of stunting 18
1.3. Morphological features of EE as seen under the dissecting microscope 26
1.4. High definition endoscopic images of small intestinal mucosa in EE 27
1.5. Typical histological features of the proximal small intestine in EE 27
1.6. The MTOR complexes and their constituent proteins 51
1.7. Integration of global cellular status signals by MTORC1 52
1.8. Overview of MTORC1 signalling 52
1.9. MTORC1 activation at the lysosomal surface 54
1.10. Downstream effects of MTORC1 activation 59
1.11. Hypothesis 72

Chapter 2.

2.1. Study flow chart 79

Chapter 3.

3.1. Example of adequately orientated section 101
3.2. Example of computer-aided morphometry 101
3.3. Confocal microscopy principle 103
3.4. The Pentax integrated CLE system 104
3.5. Examples of inadequate CLE images rejected for assessment 109
3.6. Use of CLE to assess intestinal barrier dysfunction 110
3.7. Examples of bizarre enteropathic mucosa identified by CLE *in vivo* 111
3.8. Principle of compensation 113
3.9. Effect of methanol concentration on CD3 and phospho-4EBP1 staining 114
3.10. 4EBP1 phosphorylation kinetics 115
3.11. Phospho-4EBP1 dephosphorylation kinetics 116
3.12. 4EBP1 phosphorylation kinetics after two hour nutrient deprivation 116
3.13. Predicted emission spectra for the MTORC1 panel 122
3.14. Gating strategy for determining phospho-4EBP1 positivity 125
3.15. Incompatibility of TBET and phospho-4EBP1 fix / perm protocols 126
3.16. Gating strategy to identify T_{H1} ($CD3^+$ $CD4^+$ $TBET^+$) lymphocytes 127
3.17. Representative experiment of 7-AAD staining 129
3.18. Metabonomics method 131

Chapter 4.

4.1. CONSORT flow chart 139

4.2. Marked villus atrophy and crypt hypertrophy at baseline 143

4.3. Effect of AA supplementation on change in morphometric parameters 145

4.4. Effect of MM supplementation on change in morphometric parameters 145

4.5. Interobserver correlation between two independent blinded assessors of pathological endomicroscopic barrier defects. 146

4.6. Baseline endomicroscopic barrier dysfunction by treatment group 147

4.7. Change in endomicroscopic barrier dysfunction by intervention group 147

4.8. Interaction effect of supplements on change in endomicroscopic barrier dysfunction 148

4.9. LP $CD4^+$ MTORC1 activity before & after the supplementation period 150

4.10. Effect of supplementation on change in LP $CD4^+$ MTORC1 activity 151

4.11. Correlation between MTORC1 responsiveness and villus height after the supplementation period in active treatment groups 152

4.12. Effect of AA supplementation on lamina propria $CD4^+$ T cell nutrient responsiveness post-intervention 155

Chapter 5.

5.1. Correlation between LP $CD4^+$ lymphocyte MTORC1 responsiveness and proportion of LP $CD4^+$ T_{H1} lymphocytes in HIV negative participants 159

5.2. Bimodal phospho-4EBP1 pattern in lamina propria $CD4^+$ lymphocytes in response to nutrient stimulation in vitro 161

5.3. 7-AAD staining demonstrating that all LP $CD4^+$ cells were viable before fixation and fully permeabilised after fixation & permeabilisation 162

5.4. Correlation between peripheral $CD4^+$ count and the proportion of $CD4^+$ lamina propria T lymphocytes in HIV positive participants 162

5.5. Proportion of $CD4^+$ lamina propria T cells according to HIV status 163

5.6. Correlation between serum LPS and morphometry at baseline 166

5.7. Serum levels of sCD14 according to HIV status 167

5.8. OPLS-DA loadings plots of metabolites associated with supplementation 169

5.9. OPLS-DA loadings plots of metabolites associated with MTORC1 activity 170

5.10. Serum GLP2 concentrations according to HIV status 173

5.11. Correlation between serum CRP and body fat percentage at baseline in HIV negative participants 175

LIST OF TABLES

Chapter 1.

1.1. Effect of interventions with 99% at-risk population coverage on stunting prevalence at three years 22

1.2. Selection of biomarkers previously used in studies of EE 39

1.3. Small bowel enteropathies 42

1.4. MTOR complex 1 and 2 constituent proteins and their functions 51

1.5. Summary of main effects of MTORC1 signalling in immune cells 62

Chapter 2.

2.1. Assessment and visit schedule 80

2.2. Amino acid and matched placebo composition 84

2.3. Multiple micronutrient supplement composition 86

Chapter 3.

3.1. Watson score for CLE assessment of intestinal barrier dysfunction 105

3.2. Antibody panel for the MTORC1 activity assay 121

3.3. Antibodies used for MTORC1 assay 124

3.4. Antibody panel for T_{H1} assay 127

3.5. Antibodies used for T_{H1} assay 128

3.6. Experimental details for ELISAs 132

3.7. Spearman rank correlations between 3 different measurements of BMI 134

Chapter 4.

4.1. Non-pathogenic parasites on stool microscopy 139

4.2. Baseline characteristics of the study participants at randomisation by treatment group 140

4.3. Baseline characteristics in per-protocol participants 141

4.4. Reasons for withdrawal from the study 142

4.5. Mean baseline morphometric parameters by intervention group 143

4.6. Effect of AA supplementation on change in morphometric parameters 144

4.7. Effect of MM supplementation on change in morphometric parameters 144

4.8. Endomicroscopic barrier dysfunction at baseline 146

4.9. Proportion of $CD4^+$ lamina propria lymphocytes responding to in vitro nutrient stimulation at baseline 149

4.10. Change in lamina propria $CD4^+$ MTORC1 signalling by intervention 149

4.11. Correlations between different measurements of MTORC1 activity 149
4.12. Baseline characteristics in HIV positive and negative participants 153
4.13. Baseline parameters in HIV positive and negative participants 154
4.14. Correlations between baseline parameters 156
4.15. Correlations between primary outcomes 156
4.16. Adverse events 157

Chapter 5.
5.1. MTORC1 activity according to T lymphocyte type 159
5.2. MTORC1 activity markers in $CD4^+$ compared to $CD8^+$ cells, according to HIV status 160
5.3. Maximal MTORC1 activity according to activation status 160
5.4. HIV negative: maximal phospho-4EBP1 160
5.5. Proportion of activated lymphocytes before and after supplementation 164
5.6. Serum levels of sCD14, LPS and CRP at baseline 165
5.7. Change in LPS, sCD14 and CRP over time & effect of supplementation 165
5.8. Serum levels of sCD14, LPS and CRP according to HIV status at baseline 166
5.9. Significant metabonomic changes in three active treatment groups compared to placebo 167
5.10. Significant metabonomic changes in treatment groups over time 168
5.11. Differentially excreted urinary metabolites correlated with post-treatment villus height and post-treatment MTORC1 responsiveness 168
5.12. Significant metabolites identified through metabonomic analysis: putative biological roles 172
5.13. Effect of AA or MM supplementation on fasting serum GLP2 levels 173
5.14. Baseline GLP2 levels by HIV status 173
5.15. Baseline anthropometric characteristics by intervention group. 174
5.16. Effect of intervention on body composition 174
5.17. Baseline nutritional characteristics by HIV status 175

LIST OF ABBREVIATIONS

4EBP1	(Eukaryotic initiation factor) 4E binding-protein 1
AA	Amino acids (supplementation)
ADP	Air displacement plethysmography
BCAA	Branched chain amino acid
CD	Crypt depth
CLE	Confocal laser endomicroscopy
CRF	Case reporting form
CRP	C-reactive protein
DMEM	Dulbecco's modified Eagle medium
EAA	Essential amino acid
EE	Environmental enteropathy (synonymous with EED)
EED	Environmental enteric dysfunction (synonymous with EE)
GLP2	Glucagon-like peptide 2
HAZ	Height-for-age *Z* score
HBSS	Hank's buffered salt solution
IFNγ	Interferon-gamma
IUGR	Intra-uterine growth retardation
LAL	*Limulus* amoebocyte lysate
LBP	LPS-binding protein
L:M	Lactulose : mannitol recovery ratio
LMIC	Low and middle income country
LP(MC)	Lamina propria (mononuclear cell)
LPS	Lipopolysaccharide
MM	Multiple micronutrients (supplementation)
MTOR	Mechanistic (previously mammalian) target of rapamycin
MTORC1	Mechanistic target of rapamycin complex 1
MTORC2	Mechanistic target of rapamycin complex 2
MUAC	Mid-upper arm circumference
OPLS-DA	Orthogonal partial least squares – discriminant analysis
p4EBP1	Phosphorylated eukaryotic initiation factor 4E binding-protein 1
P70S6K1	70kDa ribosomal protein S6 kinase 1
PACTR	Pan-African Clinical Trials Registry
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
RPMI	Roswell Park Memorial Institute medium
rS6	Ribosomal protein S6
RUTF	Ready-to-use therapeutic food
sCD14	Soluble CD14
TJ	Tight junction
TNFα	Tumour necrosis factor-α
UNZABREC	University of Zambia Biomedical Research Ethics Committee
UTH	University Teaching Hospital, Lusaka, Zambia
VA	Villus cross-sectional area
VH	Villus height
VW	Villus width
WAZ	Weight-for-age *Z* score
WHO	World Health Organisation
WHZ	Weight-for-height *Z* score
ZAMRA	Zambia Medicines Regulatory Authority

CHAPTER 1

INTRODUCTION

1.1 Stunting and its consequences in the developing world

Stunting (low height-for-age) affects nearly 180 million children under the age of 5 worldwide, living almost exclusively in the developing world (Black et al., 2008; de Onis et al., 2012). Improvements in living standards have resulted in the overall prevalence of stunting nearly halving from almost 50% in 1980 to just over 25% in 2010 (de Onis et al., 2012, 2000). These improvements are largely due to impressive gains in Asia; the prevalence of stunting in sub-Saharan Africa has remained stubbornly unchanged at about 40% (de Onis et al., 2012, 2000; Figure 1.1). In Zambia, for example, 40.1% of under-fives are stunted (height-for-age *Z* score –2 or greater) and 17.2% are severely stunted (height-for-age *Z* score –3 or greater) (Central Statistical Office, 2014).

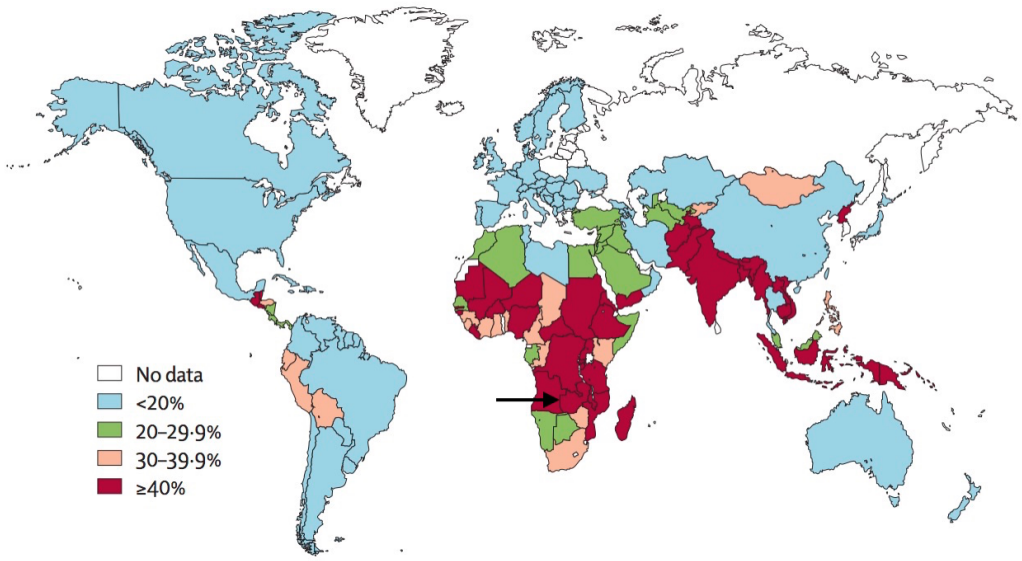

Figure 1.1. Global prevalence of stunting in children under the age of 5. Zambia, a land-locked country in southern Africa, is highlighted. Modified from Black et al., 2008.

Childhood stunting has deleterious lifelong and intergenerational effects on morbidity, mortality, and economic productivity (Dewey and Begum, 2011; Richard et al., 2013), and therefore has consequences on the individual and population level (Figure 1.2). These include

- Increased risk of metabolic syndrome (Victora et al., 2008)
- Increased risk of coronary artery disease (Stein et al., 1996)
- Increased maternal and perinatal morbidity and mortality (Black et al., 2008; Özaltin et al., 2010; World Health Organization, 1995)
- Impaired cognitive development and reduced educational achievement (Victora et al., 2008)
- Increased susceptibility to immunisable infectious disease in childhood due to reduced immunisation effectiveness (Victora et al., 2008)
- Reduced life expectancy in childhood, with a greater than four-fold risk of death in childhood in those with HAZ ≥ –3 (long-term data are not yet available due to the immaturity of the relevant cohort studies) (Black et al., 2008; Özaltin et al., 2010)
- Reduced earnings in adulthood, even when corrected for educational level (Victora et al., 2008)
- Increased risk of stunting in children of stunted mothers (Gough et al., 2016; Victora et al., 2008)

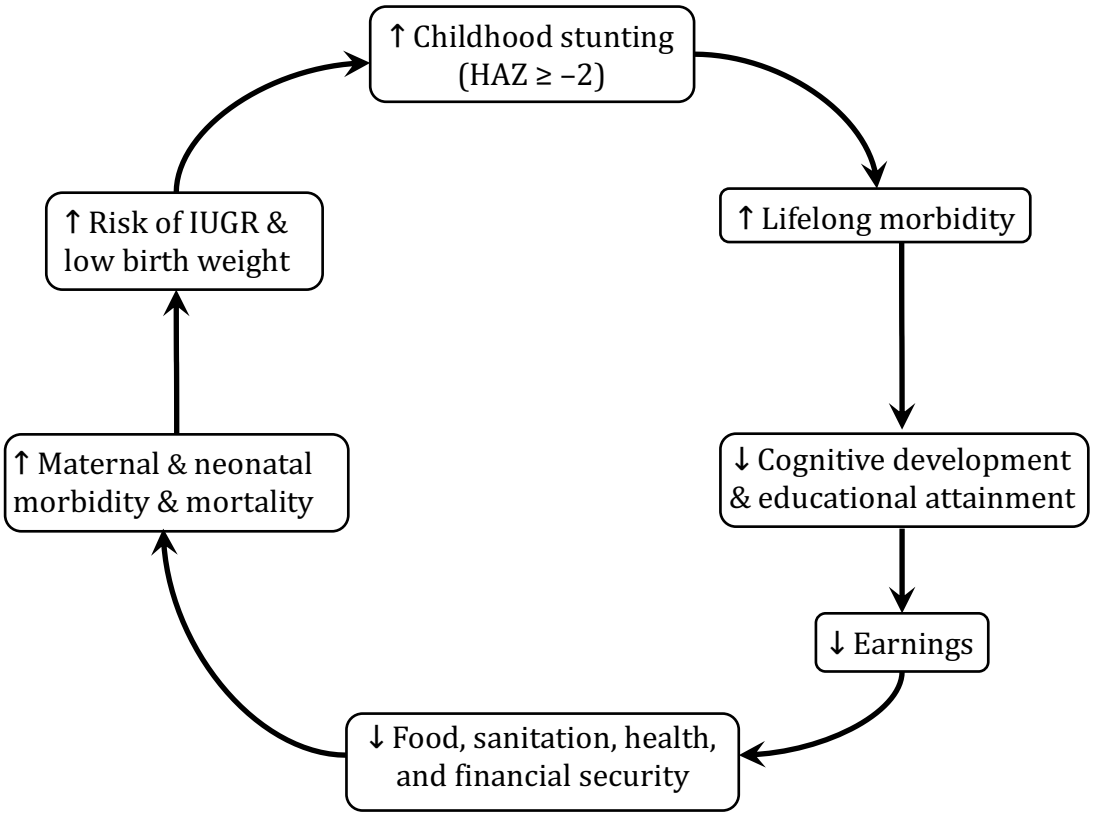

Figure 1.2. Long term and intergenerational consequences of stunting. HAZ, Height-for-age Z score.

Stunting and its sequelae are a consequence of multiple interacting deleterious pathological processes. Stunting may therefore be used as a biomarker to measure the effectiveness of interventions aimed at improving adult, peri-conception, pregnancy and early childhood outcomes.

1.2 Causes of stunting: undernutrition, infection, WASH, and environmental enteropathy

1.2.1 Introduction

Several lines of evidence suggest that the aetiology of stunting is multifactorial, but remains incompletely explained. Populations affected by stunting are also characterised by unhygienic living environments without access to clean water and sanitation, by food insecurity (with varying degrees of overt and subclinical malnutrition), by high rates of exposure to gastrointestinal pathogens in particular, and by a high prevalence of HIV. Numerous interventional trials have attempted to improve stunting and its sequelae by targeting these presumed causes; unfortunately, none have been successful.

1.2.2 Inadequate protein-energy and / or micronutrient intake

The prevalence of stunting (low height-for-age *Z* score, HAZ) does not predict the prevalence of wasting (low weight-for-height *Z* score, WHZ), and countries with similar stunting prevalences may have vastly different prevalences of wasting (Black et al., 2008), suggesting that dietary intake in itself is not sufficient to explain stunting.

1.2.2.1 Effects of protein-energy supplementation

Impoverished populations, even if not overtly malnourished, tend to subsist on a diet that includes poor protein diversity and which is relatively carbohydrate

heavy. A meta-analysis of complementary feeding supplements in food insecure populations, with or without a nutrition education programme, suggested improved HAZ scores by 0.41 *Z* scores (Bhutta et al., 2008).

1.2.2.2 Effect of micronutrient supplementation

Zinc supplementation has a small beneficial effect on stunting on infants and older children (0.35 *Z*), with an associated reduction in diarrhoeal episodes and death in this age group (Bhutta et al., 2008). Multiple micronutrient supplementation in pregnancy provides a modest reduction in risk of low birthweight (OR 0.83) and small for gestational age (OR 0.92) (Bhutta et al., 2008; Haider and Bhutta, 2015). Iron and vitamin A supplements do not improve growth, although vitamin A supplementation reduces childhood mortality (Bhutta et al., 2008). Iron supplementation appears to increase the risk of infectious complications including diarrhoea, and death due to (undertreated) malaria (Sazawal et al., 2006).

1.2.3 Infectious disease burden

The number of diarrhoeal episodes is a risk factor for stunting (Black et al., 2008). It was initially postulated that stunting was the result of frequent acute infections, enteric or otherwise, which would limit the potential for catch-up growth normally observed in otherwise healthy children. However it was clearly demonstrated in the early 1990s in children from the Gambia that abnormal intestinal permeability persisted even during periods of asymptomatic good health, and that growth velocity was inversely correlated to intestinal permeability (as assessed by

lacutulose:mannitol recovery ratio) (Lunn et al., 1991). Consistent with this, a pooled analysis of the effect of diarrhoeal episodes on stunting at two years of age found evidence of a small height deficit only (Richard et al., 2013): in other words, children exhibit catch-up growth in between diarrhoeal episodes but remain stunted, and diarrhoeal illness *per se* is not causative.

Rates of asymptomatic helminth carriage in stunted populations is high. However deworming treatment in children up to the age of 16 results in very small improvements in height (0.14cm after 1 year) and weight (0.24kg after 1 year) (Bhutta et al., 2008).

1.2.4 Water, sanitation, and hand washing (WASH)

A programme of multiple hygiene interventions has been estimated to reduce the risk of diarrhoeal episodes by 30% (Bhutta et al., 2008). This will have a direct and significant effect on reducing childhood mortality from diarrhoeal illness, but is estimated to have a minor reduction of up to 2.4% on stunting prevalence (Bhutta et al., 2008). This has subsequently been confirmed in large randomised controlled trials (Luby et al., 2018; Null et al., 2018).

1.2.5 The need for novel mechanisms and interventions

Correction of undernutrition, micronutrient supplementation, control of infectious disease, and hygiene / sanitation interventions all have important roles in reducing short-term childhood mortality, but have been shown to have only minor effects on

childhood stunting and its lifelong consequences. It is estimated that even with 99% coverage with an aggressive combination of interventions to reduce disease burden (e.g. deworming; mosquito net provision), improve sanitation (e.g. hand washing interventions), improve general nutrition (e.g. breast feeding promotion), and correct micronutrient deficiencies, just over 1/3 of stunting is preventable (Bhutta et al., 2008; Table 1.1). Although highly prevalent in stunted populations, these problems therefore do not explain the high prevalence of stunting observed, implicating other factors in its pathogenesis. In recent years, attention has therefore shifted towards environmental enteropathy, which is now thought to mediate many of the pathological processes resulting in stunting.

Intervention (99% coverage)	**Relative reduction in stunting prevalence at 3 years**
General nutrition interventions	15.5%
Micronutrient interventions	17.4%
Disease control interventions	2.7%
All interventions combined	**35.5%**

Table 1.1. Effect of interventions with 99% at-risk population coverage on stunting prevalence at three years. Modified from Bhutta et al., 2008.

1.3 Environmental enteropathy (EE)

1.3.1 Environmental enteropathy: an acquired, reversible disorder of poverty

A number of investigations into overt, symptomatic diarrhoea and malabsorption in the tropics in the early 1960s used apparently asymptomatic, healthy and well-nourished adults and children from the same population as control subjects. Unexpectedly, these studies uniformly identified a high prevalence of abnormal intestinal absorptive capacity and permeability (as measured by urinary sugar recovery) and / or varying degrees of villous blunting, crypt hypertrophy, villous fusion, and mucosal inflammation) in these controls (Lindenbaum et al., 1972).

Studies in American soldiers and Peace Corps volunteers stationed in Thailand (Keusch et al., 1972) and in Peace Corps volunteers in modern-day Bangladesh (Lindenbaum et al., 1966b) showed that the condition was acquired, and that these abnormalities were similar to those observed in the native population (Lindenbaum, 1968). Furthermore, *post mortem* examination of foetal and neonatal intestine showed that these abnormalities were not present *in utero* or at birth, and only became apparent during early childhood (Chacko et al., 1969). Similarly, although not present in neonates, persistent abnormalities in small intestinal absorption and permeability were observed from as little as 3 months of age, and these abnormalities were correlated with growth faltering (Lunn et al., 1991).

These changes were reversible, as demonstrated by prospective assessment of small bowel histology and absorption. Peace Corps volunteers who had lived in India or Pakistan returned to histological and absorptive normality, usually within

two years after returning to the United States (Lindenbaum et al., 1972, 1971). Furthermore, intestinal structure and function in adult students from endemic areas moving to the United States to study also normalised with time (Gerson et al., 1971).

Based on these initial studies and others which were exclusively conducted in the tropics, the condition was labelled 'tropical enteropathy'. However, an extensive worldwide study clearly demonstrated that the observed abnormalities were not observed in some affluent, 'tropical' populations (such as Singapore and Qatar) (Menzies et al., 1999), and the condition is therefore more correctly termed 'environmental enteropathy' (EE) or 'environmental enteric dysfunction' (EED). EE is strongly associated with stunting, and is highly prevalent in stunted populations irrespective of climate, and in particular is associated with household wealth (Kelly et al., 2004b; Menzies et al., 1999). For example, in a poor urban community in Zambia where the prevalence of childhood stunting is approximately 40%, EE is virtually universal among overtly healthy adults (Kelly et al., 2004b).

Increasing evidence suggests that the severity of EE, as well as being reversible, has a seasonal component. In general, populations with EE live in tropical or sub-tropical climates with well-defined wet and dry seasons. Studies from enteropathic populations in urban Zambia (Kelly et al., 2004b) suggest a deterioration in histological features and sugar permeability / absorption during the wet season. Heavy rains in populations with inadequate sewerage and sanitation are at high risk of potable water contamination and faecal-oral transmission of intestinal infections. Furthermore, undernutrition may be exacerbated during this period: in the rainy season crops are growing and are unavailable for consumption (for this

reason the rainy season is colloquially known as the 'hungry season' in populations whose nutritional intake is precarious and heavily reliant on subsistence). The role of nutrition in EE is discussed in section 1.3.4 below.

Multiple well conducted prospective cohort studies provide good evidence that environmental conditions, in particular access to clean water and good sanitation, are implicated in the severity of EE. In rural Bangladesh for example, children from 'clean' households (defined using objective markers of water cleanliness and sanitation infrastructure) had significantly increased linear growth, reduced lactulose:mannitol ratios (indicating reduced permeability and improved absorption) , and reduced serum anti-endotoxin IgG compared to children from 'dirty' households, after adjustment for confounders (Lin et al., 2013a).

1.3.2 Environmental enteropathy: features, pathophysiology, and possible aetiologies

1.3.2.1 Histological abnormalities and deficits in intestinal absorptive capacity

The original reports of EE described a spectrum of architectural abnormalities in proximal small bowel biopsies (Figure 1.3), progressing from blunted but otherwise normal 'finger-like' villi through 'leaves', ridges, and convolutions and finally to atrophy as the severity of the enteropathy increased (Schenk et al., 1972, 1968; Schenk and Klipstein, 1972). With modern high definition endoscopes, these abnormalities which previously could only be appreciated under the dissecting microscope can now be readily visualised *in vivo* (Figure 1.4, p.27). Histologically, these correspond to varying degrees of crypt hypertrophy, villous atrophy, and

villous fusion (Lindenbaum et al., 1966a; Figure 1.5), resulting in a reduction in proximal small bowel absorptive area.

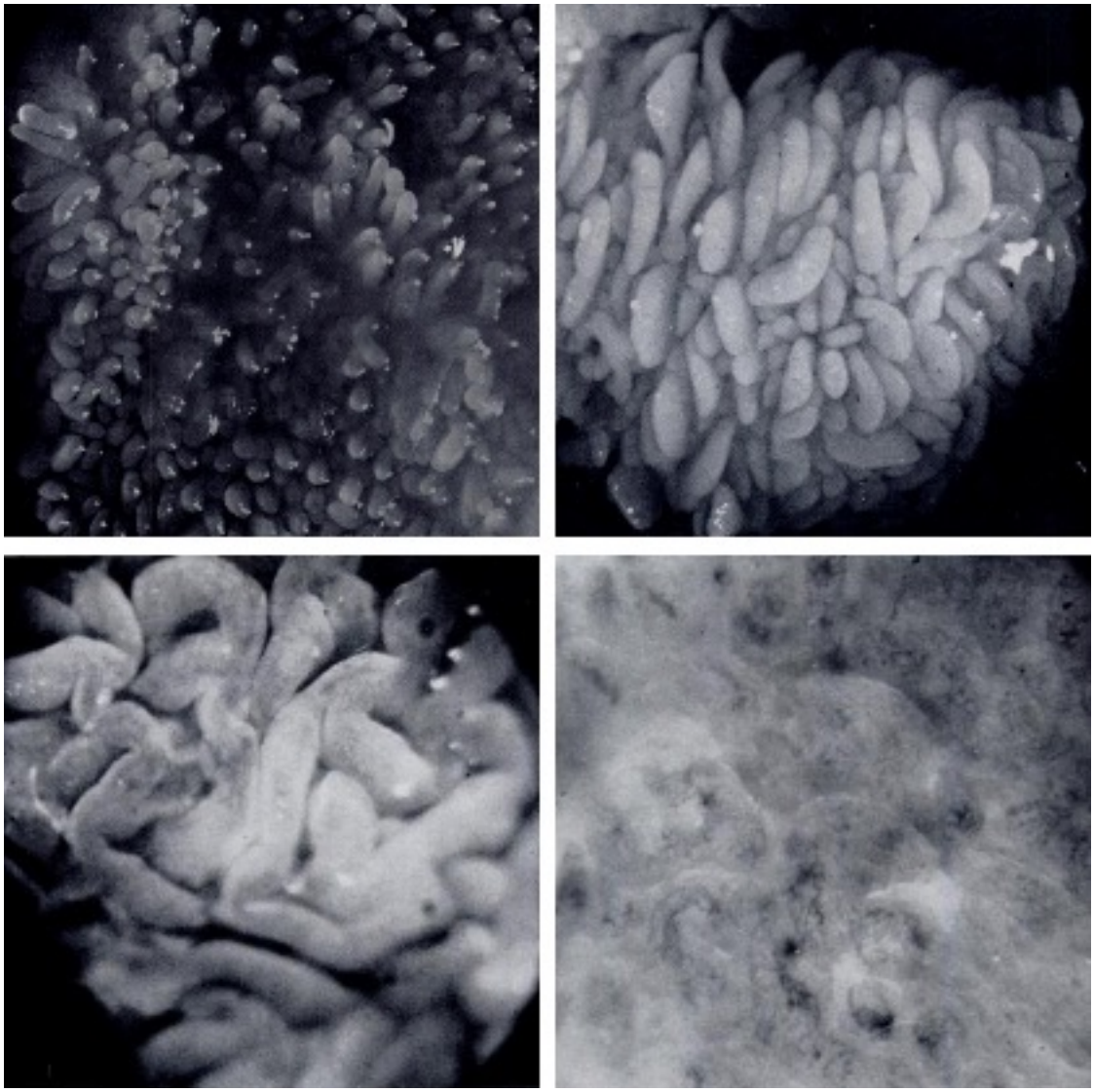

Figure 1.3. Morphological features of EE as seen under the dissecting microscope in increasing order of severity. Top left, normal villi. Top right, predominant leaves (fused villi). Bottom left, ridges with convolutions. Bottom right, subtotal atrophy. Taken from England, 1968; Sheehy et al., 1968.

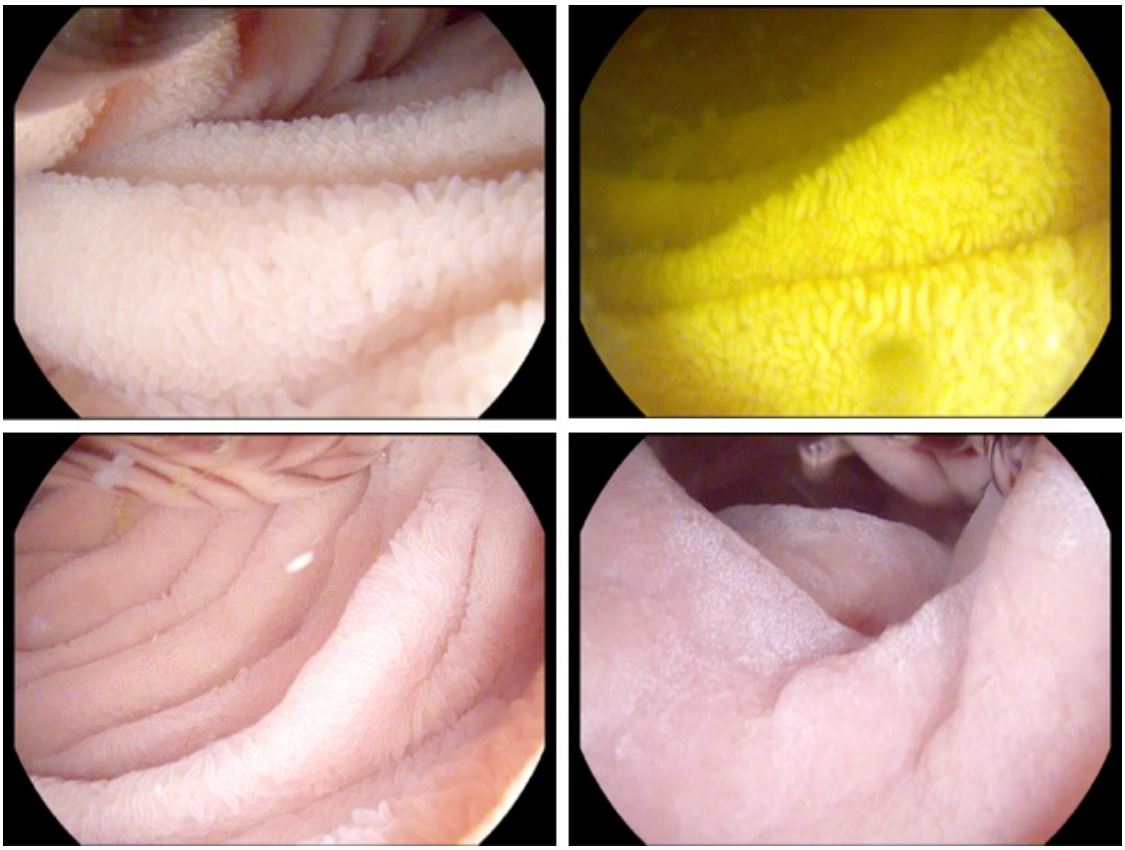

Figure 1.4. High definition endoscopic images of proximal small intestinal mucosa in EE, demonstrating increasingly severe enteropathic changes. Top left, relatively normal mucosa in an unaffected affluent patient. Top right, predominant leaves. Bottom left, ridges with convolutions. Bottom right, subtotal atrophy.

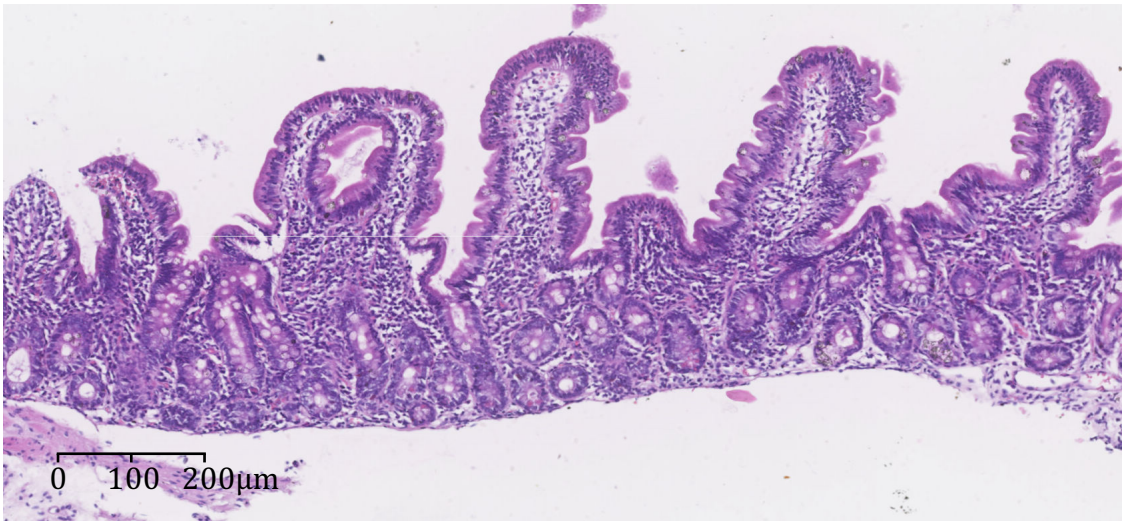

Figure 1.5. Typical histological features of the proximal small intestine in EE. Fused villi, crypt hypertrophy, villous atrophy, and gross lamina propria inflammatory infiltrate are evident. H&E section of an endoscopic biopsy taken from a participant in the current study.

Although these abnormalities are observed only in the proximal small bowel, they result in significantly reduced absorptive capacity. Xylose recovery, for example, is reduced by approximately 50% in asymptomatic adults (Kelly et al., 2004b; Menzies et al., 1999). However, the clinical relevance of this is uncertain. An early study in Bangladeshi children showed that although xylose malabsorption in children with EE is correlated with carbohydrate malabsorption, an estimated >90% of ingested carbohydrate was still absorbed overall and no effect was observed on protein or fat absorption (Brown et al., 1981).

Conversely, zinc homeostasis is perturbed in EE, where there is impaired capacity to resorb endogenous zinc in the distal small bowel regardless of zinc status, resulting in net loss of zinc (Manary et al., 2010). There is a strong positive correlation between the severity of enteropathy as assessed by lactulose : mannitol ratio and excretion of endogenous zinc (and an inverse relationship to resorption) (Manary et al., 2010).

It is unknown to what extent the endoscopic, microscopic, and histological abnormalities observed in EE correlate with each other. In particular, the correlation between endoscopic features and histology and / or barrier dysfunction has not been established.

1.3.2.2 Intestinal barrier dysfunction

The healthy intestinal mucosa provides a physical and functional barrier against constant microbial and dietary antigen exposure, while at the same time mediating the uptake and absorption of most ingested nutrients and water. Impairment or

dysregulation of barrier function is characterised by increased permeability, which can result in both the translocation of microbes and microbial products into the host and loss of nutrients and water into the gut lumen.

An impaired small intestinal mucosal barrier is a canonical feature of EE. Increased intestinal permeability (as measured by urinary sugar recovery assays) in EE is correlated with pathological abnormalities of intestinal barrier function and growth. Growth velocity is inversely correlated to intestinal permeability (Lunn et al., 1991) and positively correlated with bacterial translocation as measured by plasma LPS levels (Campbell et al., 2003a). Chronic endotoxaemia in EE depresses IGF1 secretion, providing a mechanism of growth suppression and stunting (Prendergast et al., 2014).

Barrier dysfunction may result from physical defects in the mucus barrier or epithelium, or functional defects in permeability or mucosal immune function.

Dysfunction of the mucus barrier

The mucus barrier provides a physical, biochemical and anti-microbial barrier to microbiological insults. The small intestinal mucus layer is porous to allow the uptake of fluid, electrolytes and nutrients. The mucus layer is continuously replaced, resulting in the expulsion of trapped bacteria. Bacterial invasion through this layer is further limited by a diffusion gradient of secreted antimicrobial peptides (AMP) and IgA (Johansson and Hansson, 2016). The mucus barrier also moulds the commensal microbiota through the excretion of anti-microbial immunoglobulins and peptides, and in the expression of glycoproteins which can

promote the proliferation and diversification of anti-inflammatory bacterial species.

It is increasingly recognised that mucus composition and production is tightly regulated, but clinical consequences of mucous barrier defects have not been well studied. Mucin defects are sufficient to produce colitis in mice (Heazlewood et al., 2008), and may be a risk factor for the development of ulcerative colitis (UC) in humans (Visschedijk et al., 2016). Although the mucus barrier in EE and other conditions of undernutrition have not been specifically studied, diffuse intestinal glycosaminoglycation defects occur in kwashiorkor (Amadi et al., 2009) which is likely to include mucus modifications. The microbial implications of this are unknown.

The antimicrobial properties of mucus in EE is discussed in section below.

Defects in transcellular epithelial barrier integrity

All intestinal epithelial cell types appear to be generated from intestinal stem cells (ISC), which reside in stereotyped positions (the '+4' position) in the stem cell niche located at the base of intestinal crypts. On differentiating, epithelial cells migrate upwards out of the crypt towards the intestinal luminal surface (in the colon) or villus (in the small intestine), with the exception of Paneth cells, which remain near the base of the small intestinal crypts (van der Flier and Clevers, 2009). Senescent epithelial cells at the villous tips undergo apoptosis and are shed into the intestinal lumen. This is an active process, where adjacent cells 'squeeze out' the dying cell while re-establishing tight junction integrity between the new

neighbours, thus preventing any epithelial breach. This has been demonstrated most elegantly using confocal laser endomicroscopy (CLE) *in vivo* (Kiesslich et al., 2012).

Most obviously, defects in epithelial integrity, renewal or maturation result in erosions and ulcers. These are physical breaches in the epithelial barrier which may be apparent macroscopically (for example *in vivo* at endoscopy), microscopically *in vivo* using CLE (Kiesslich et al., 2007), or histologically. An elegant CLE study in a mouse model using fluorophore-labelled dextrans has demonstrated that *in vivo* microscopic epithelial defects (visible by CLE but not endoscopically) result in the bidirectional leakage of macromolecules similar in size (MW 10kDa) to physiologically relevant microbial proteins such as LPS. Interestingly, these epithelial defects may result in influx, efflux, both simultaneously, or none (Kiesslich et al., 2012). We have recently described similar defects in EE (Kelly et al., 2016).

Strikingly, even very significant mucosal defects (such as those created therapeutically for mucosal resections for polyps) in otherwise normal guts do not normally result in disease, due to poorly understood immune regulatory crosstalk (Turner, 2009).

Defects in paracellular epithelial barrier integrity

Under normal circumstances, paracellular flux is tightly regulated and is the primary determinant of trans-epithelial transport due to the relative impermeability of transcellular pathways (Shen et al., 2009; Turner, 2009).

Paracellular permeability is determined by the composition and function of tight junctions (TJ), apical protein complexes which together with adherens junctions and desmosomes bind epithelial cells together. TJ complexes include claudins, occludins, zonulins and myosin light chain kinase (Turner, 2009).

Intestinal TJ demonstrate variable degrees of leak which allows the non-selective passage of molecules including proteins up to about 50Å (5nm) in the crypt and 6Å (0.6nm) at the villus tip (Shen et al., 2009). This 'leak pathway' demonstrates size but not charge selectivity and is modified by inflammatory signals including T cell activation, TNFα, NFκB and IFN (Clayburgh et al., 2005; Tang et al., 2010; Turner, 2009; Watson et al., 2005; Zolotarevsky et al., 2002). In contrast, tight junction pores, formed by claudins, form a second 'charge selective pathway' which limits the passage of molecules larger than approximately 4Å (0.4nm). The number and function of pores also appears to be regulated by immune signalling (Turner, 2009). TJ defects have been implicated in contributing to disease severity in a murine model of colitis (Su et al., 2009) , and TJ pores (and thus permeability) may be upregulated in inflammatory bowel disease (Heller et al., 2005; Zeissig et al., 2007).

Claudins and other TJ proteins are responsible for sealing epithelial gaps associated with physiological apoptotic cell shedding events (Marchiando et al., 2011). We have previously demonstrated that, although these processes appear to be intact in individuals with EE, the extent of the epithelial defects means that the claudin system is unable to seal the epithelial defects (Kelly et al., 2016).

Abnormalities of the adaptive mucosal immune system

The mucosal immune compartment provides an immunological barrier to reinforce the physical barriers provided by the epithelium and mucus layers. Innate and adaptive arms both play important roles, with deficits resulting in either suppressed mucosal defence (allowing microbial invasion) or pathologically enhanced inflammation (resulting in maladaptive host tissue damage). The regulation of pro- and anti-inflammatory responses in the face of continuous exposure to dietary and microbial antigens are exceptionally complex and remain incompletely understood, including in EE.

Lamina propria (LP) lymphocytes are normally predominantly antigen-experienced $CD4^+$ T cells, with a smaller proportion of $CD8^+$ cells also present (Shale et al., 2013). LP lymphocytes consist of circulating effector memory T cells, tissue resident T cells and, in the secondary lymphoid organs of the intestine including Peyer's patches and lymphoid follicles, naïve and central memory T cells (Sallusto et al., 2004; Schenkel and Masopust, 2014). The specific makeup and contributions of these different T cell populations in human health and disease is largely unknown.

The immunological features of EE are not well documented and studies are conflicting. The inflammation observed in EE is IFNγ predominant, indicating a T_{H1}-type response (Campbell et al., 2003a, 2003b; Veitch et al., 2001). In children with EE there is a four-fold increase in intra-epithelial lymphocytes (IEL), and a ten-fold increase in activated cytotoxic T lymphocytes (Campbell et al., 2003b). However in two studies in adults, there were no differences in IEL density between adults with EE and unaffected controls (Veitch et al., 2001; Wood et al., 1991).

The immunological characteristics of the LP compartment in EE have not been studied extensively. A study in Gambian children with EE suggested that LP T lymphocyte density increased with worsening nutritional status (WHZ), and the $CD4^+$ and $CD8^+$ cells were present in roughly equal proportions, in contrast to 'normal' lamina propria in UK controls where the $CD4^+$ to $CD8^+$ ratio was 2:1 (Campbell et al., 2003b). There were also elevated densities of B lymphocytes compared to UK controls (Campbell et al., 2003b). In adults, LP $CD3^+$ cells are predominantly activated (median 87% HLA-DR^+ in Zambians with EE, compared to 65.5% in white South African controls) (Veitch et al., 2001).

LP plasma cells secrete immunoglobulins into the mucosa, where immunoglobulins (predominantly but not exclusively IgA) are actively transported across the epithelium and secreted into the mucus barrier (Kaetzel et al., 1991). This component of the adaptive immune response protects against pathogenic bacteria, and also promotes the establishment of the host commensals. Studies of secreted intestinal IgA in malnourished children are conflicting (Rytter et al., 2014); the role of IgA in asymptomatic individuals with EE has not been studied.

Paneth cell and antimicrobial peptide dysfunction

Antimicrobial peptides (AMP) are secreted into mucus by Paneth cells in the small intestinal crypt. A number of peptides are secreted which have powerful antimicrobial properties, and which play an important role in regulating the host microbiota.

Paneth cells granule depletion is more prevalent and AMP expression is downregulated in EE (Dhaliwal et al., 2003; Kelly et al., 2004a), and in HIV negative adults is inversely correlated with the histological severity of enteropathy (Kelly et al., 2006). Intriguingly, nutrient deprivation in an animal model results in autophagy of Paneth cells and impaired AMP production (Hodin et al., 2011).

1.3.2.3 The microbiota and dysbiosis in environmental enteropathy

The complex reciprocal relationship between the microbiota, diet and mucosal inflammation has only recently been appreciated, and remains poorly understood. The microbiota has been implicated as being a causative agent in a number of conditions characterised by intestinal inflammation including inflammatory bowel disease and necrotising enterocolitis. It is now also accepted that the intestinal microbiota also has important effects on nutrient harvest and mucosal immune homeostasis. The study of the effects of the microbiota in undernutrition and overt malnutrition is still in its infancy.

Microbiological insights

In kwashiorkor, the microbiota is immature and is less responsive to therapeutic nutritional supplementation (Smith et al., 2013). Compared to Western subjects, asymptomatic children and adults living in EE-endemic areas have significantly different microbiota, as assessed by faecal 16S rRNA sequencing (De Filippo et al., 2010; Lin et al., 2013b). In a study in Bangladesh, the microbiota was also less stable over time (Lin et al., 2013b), a finding also observed in a Malawian

kwashiorkor study (Smith et al., 2013). In rural Burkina Faso, Bacteroidetes are significantly enriched and Firmicutes are depleted (De Filippo et al., 2010), mirroring the pro-inflammatory microbiota observed in IBD. Conversely, in urban Bangladesh, the opposite is true (Lin et al., 2013b). These studies did not investigate the role of the microbiota in enteropathy or stunting, so the significance of these findings is unclear.

Non-pathogenic microbes are frequently isolated from stool samples in individuals with EE. In one study in urban Zambia, for example, *Citrobacter rodentium* was overrepresented (Kelly et al., 2004b). This bacterium, although not classically pathogenic in humans, is pro-inflammatory and induces ulcerative colitis in murine models. Non-pathogenic intestinal parasites in the stool of individuals with EE are also frequently identified, and serve as a marker of unsanitary living conditions. The possible role of these microbes, generally considered to be non-pathogenic, in promoting and perpetuating the intestinal inflammation observed in EE is unknown; furthermore, the contribution of non-bacterial microbes (especially parasites) to the microbiota has not been studied.

Dietary intake, the microbiota, and nutrient harvest

Populations where EE and stunting are prevalent also have a diet low in animal fat and protein but high in starch, fibre, and plant polysaccharides, resulting in major differences in the microbiota compared to a 'Western' diet (De Filippo et al., 2010; Grześkowiak et al., 2012; Turnbaugh et al., 2009; Wu et al., 2011; Yatsunenko et al., 2012). The resulting microbiota influences body composition and nutrient energy

harvest (Jumpertz et al., 2011; Turnbaugh et al., 2006), but may also promote the establishment of pro- or anti-inflammatory microbes (Devkota et al., 2012).

Effect of the immune response on the microbiota

Microbes may promote pro- or anti-inflammatory responses in the host. In turn, the host's immunological response in conjunction with dietary intake can modulate the microbiota. Paneth cell degranulation and antimicrobial peptide release occurs in response to a number of immunological and microbiological triggers (Bevins and Salzman, 2011a). Interestingly, commensal bacteria are relatively resistant to antimicrobial peptides (Cullen et al., 2015); the effect of the subtle abnormalities of Paneth cell function observed in EE (Dhaliwal et al., 2003; Kelly et al., 2004a, 2006) on the microbiota is unknown.

The prevalence of helminth infection in stunted populations is high. Although the specific immunological consequences of this in EE have not been studied, helminthic infection results in a predominant T_{H2}-type response, and also results in a significantly impaired T_{H1} / T_{H17} and innate immune response to GI pathogens including *Salmonella* Typhimurium and *C. rodentium* in mice models (Chen et al., 2005; Su et al., 2014).

Modulation of the gut barrier by the microbiota

Tight junctions are the target of many microbial virulence factors. Enteropathogenic and enterohaemorrhagic *E. coli*, *Vibrio cholerae*, rotaviruses,

Salmonella Typhimurium and others produce effector molecules which disrupt TJ protein production and utilisation (König et al., 2016). 'Anti-inflammatory' bacteria such as VSL#3, *Lactobacillus rhamnosus*, and *L. helveticus* appear to promote TJ protein expression and function in animal models and *in vitro* models (Mennigen et al., 2009; Miyauchi et al., 2009; Zareie et al., 2006); presumably 'pro-inflammatory' bacteria will have the opposite effect although this has not yet been demonstrated. The immune signalling response provoked by microbial inflammation also has effects on TJ structure and function, as described above.

1.3.2.4 Biomarkers and environmental enteropathy

There is an unmet need for biomarkers in EE. A variety of blood, stool and urine markers have been trialled (Table 1.2) but none used alone or in combination are sufficiently sensitive or specific for diagnostic or prognostic purposes (Guerrant et al., 2016; Keusch et al., 2014; Prendergast et al., 2015).

More recently, metabonomic studies have begun to identify putative markers of impaired gut structure and function in EE (Claus and Swann, 2013; Farràs et al., 2018; Guerrant et al., 2016; Mayneris-Perxachs et al., 2016; Mayneris-Perxachs and Swann, 2018), but more work is required to confirm validity and applicability.

Blood abnormalities are also common but non-specific. Frequently encountered abnormalities include anaemia (macro-, normo- and microcytic) due to chronic inflammation and / or micronutrient deficiencies; elevated inflammatory markers such as CRP (generally low level, in contrast to the levels seen in severe acute malnutrition); and low plasma micronutrient levels (e.g. retinoids; zinc; iron).

Biomarker	Origin	EE domain assessed	Comment
LPS	Plasma	Intestinal permeability; microbial translocation	Bacterial product; detection in blood implies translocation
Anti-LPS IgG	Plasma	Microbial translocation	Quantifies host adaptive response to systemic LPS
sCD14	Plasma	Systemic immune activation; microbial translocation	Marker of innate immune activation; secreted by activated monocytes / macrophages in response to LPS
LBP	Plasma	Systemic immune activation; microbial translocation	Acute phase reactant in response to systemic LPS; agonist for TLR signalling and monocyte activation
MBL	Plasma	Systemic immune activation; microbial translocation	Acute phase protein; binds to bacterial carbohydrate residues
CRP	Plasma	Systemic immune activation	Non-specific marker of systemic immune activation
Citrulline	Plasma	Absorptive capacity	Biproduct of enterocyte glutamine metabolism; used as a marker of enterocyte mass
iFABP	Plasma	Intestinal barrier damage	Released from damaged enterocytes; higher levels observed in EE than in coeliac disease
α1AT	Stool	Intestinal permeability	Serum protein detectable in stool from abnormally permeable intestine
MPO	Stool	Intestinal inflammation	Secreted by activated neutrophils / monocytes / macrophages in inflamed intestine
Calprotectin	Stool	Intestinal inflammation	Secreted by activated neutrophils / monocytes / macrophages in inflamed intestine
Neopterin	Stool	Intestinal inflammation	Secreted by activated neutrophils / monocytes / macrophages in inflamed intestine
L:M (and others)	Urine	Intestinal permeability; absorptive capacity	Assesses systemic absorption of absorbable and non-absorbable sugars

Table 1.2. Selection of biomarkers previously used in studies of EE. CRP, C reactive protein; LPS, lipopolysaccharide; α1AT, α_1-anti-trypsin; LBP, LPS binding protein; MPO, myeloperoxidase; iFABP, intestinal fatty acid binding protein; MBL, mannose binding lectin; sCD14, soluble CD14; L:M, urinary lactulose : mannitol recovery ratio; TLR, toll-like receptor.

1.3.3 Co-existent causes of enteropathy: similarities & differences

Patients with EE invariably have a number of histological abnormalities including reduced VH:CD ratio due to villous atrophy and / or crypt hypertrophy, intra-epithelial lymphocytosis, and LP lymphocyte infiltration. These findings are non-specific and are observed in other small bowel enteropathies (Table 1.3, p.42). Other causes of enteropathy which are highly prevalent in populations with EE and which may coexist with it include HIV infection (asymptomatic / well controlled, as well as HIV-AIDS), helminth and other parasite infections, and severe acute malnutrition (SAM).

HIV infection results in a dramatic and early decline of LP $CD4^+$ populations. Despite the resultant systemic and mucosal activation, asymptomatic HIV infection appears to have subtle (if any) additional effects on small intestinal morphometry, absorption / permeability, and anti-microbial peptide expression in populations affected by EE (Dhaliwal et al., 2003; Kelly et al., 2004b, 2006); in other words, the primary pathophysiological mechanism of the observed defects in the intestinal barrier appears to be mediated through EE.

The relative contribution of helminth infection to the pathophysiology of EE has not been studied; however treatment with anti-helminthics may improve intestinal function (section 1.5.1 below).

Although not proven, SAM is likely to occur on a background of EE, as it invariably affects individuals from populations where the prevalence of EE is high. However, the role of EE and pre-existing barrier dysfunction in the pathogenesis of SAM is unknown. The villus atrophy / crypt hypertrophy observed in SAM appears to be

similar to 'background' EE, although functional deficits such as microbial translocation are significantly greater (Amadi et al., 2017).

Tropical sprue, although histologically similar, is an uncommon condition with overt malabsorptive symptoms which may affect individuals with EE. It is not described in sub-Saharan Africa.

Condition	Presumed aetiology	Diagnosis	Intestinal distribution	Populations affected	Villus atrophy	Mucosal inflammation	Barrier defect	Systemic inflammation	Malabsorption	Management	Mortality
EE	Undernutrition; dysbiosis	E; H; F	DJ	LMIC	+ / +++	++	+	+	+	?	(–)
Kwashiorkor	Undernutrition; dysbiosis	C	DJI	LMIC	+ / +++	++	++	++	++	Nutrition; antibiotics	+++
Marasmus	Undernutrition	C	DJI	LMIC	+ / +++	++	+	+	+	Nutrition	++
Coeliac disease	Cross–reactivity to dietary gluten	S; H	DJ	Caucasian	+ / +++	++	–	–	++	Dietary restriction	–
Tropical sprue	Infectious	C	D	Caribbean; South Asia	+ / +++	++	+/–	–	++	Antibiotics; folate	–
Crohn's	Dysregulated immunity; dysbiosis	E; C; H	(J)I	Western	+	+++	++	++	+	Nutrition; immune-modulation	–
Helminths and *Giardia*	Infectious	M; S	DJ	LMIC	+ / ++	+	+	–	+	Antimicrobials	–
HIV (early or well controlled)	Inflammatory / immune mediated	M; S	DJI	Worldwide	+	++	+	+	+	Antivirals	–
HIV–AIDS	Infectious	C; M; H; S	DJI	Predom. LMIC	+ / +++	+++	+++	+++	+++	Antivirals / antimicrobials	+++
Disuse	Lack of trophic stimuli	C; H	DJI	Western	+ / +++	+	+	–	–	Enteral nutrition	–

Table 1.3. Small bowel enteropathies. *C, clinical; M, microbiological; E, endoscopic; H, histology; F, functional tests (sugar absorption etc.); S, serological; D, duodenum; J, jejunum; I, ileum; LMIC, low and middle income country.* ***Modified from Louis-Auguste and Kelly, 2017.***

1.3.4 Case definitions and diagnostic criteria

Despite its frequently dramatic laboratory features, EE is an asymptomatic disorder (Prendergast and Kelly, 2012).

EE is primarily characterised by

1. Increased small intestinal permeability and impaired barrier function
2. Reduced proximal small intestinal absorptive area

in an asymptomatic individual living in an at-risk environment.

The similarities and differences between EE and other conditions resulting in similar abnormalities of the small bowel mucosa are outlined in Table 1.3 above, although individuals with EE may have other co-existing enteropathies.

Histological assessment of small intestinal tissue is the current gold standard method for identifying and assessing EE. However, small intestinal tissue is obtained endoscopically, a resource-intensive technology which is not widely available in the populations affected by EE. Furthermore, endoscopy with biopsies, although extremely safe, is more dangerous in paediatric populations, and is therefore not generally used in the assessment of children.

A commonly used surrogate of intestinal function is non-invasive urinary sugar recovery assays. Early studies of EE demonstrated significant abnormalities using these tests and in paediatric populations, EE is generally defined as abnormal sugar permeability / absorption in a population known to be affected by EE (Denno et al., 2014).

There are therefore no universally agreed diagnostic criteria for EE. This is due to the absence of sufficiently sensitive and specific biomarkers, and the difficulty in obtaining small intestinal and other bodily tissue from paediatric individuals. The identification of reproducible and non-invasive methods for robustly assessing the gut in EE is therefore an area of considerable interest.

1.4 Role of nutrition in environmental enteropathy

1.4.1 Nutritional deficiencies in Zambia

Subclinical as well as overt micronutrient deficiencies implicated in mucosal and systemic immunity and intestinal epithelial function, including retinols and zinc, affect nearly 20% of Zambian adults and 65% of Zambian infants (Gibson et al., 2011; Kelly et al., 2008).

Dietary protein diversity in impoverished communities tends to be limited and plant based. The staple diet in Zambia is *nshima*, a maize-based dough. Maize protein is relatively indigestible and therefore of poor quality (Joint WHO/FAO/UNU Expert Consultation, 2007). It is phytate-rich which inhibits zinc and iron absorption in particular (World Health Organization et al., 1996), and processing / production results in destruction of niacin.

1.4.2 Rationale for nutritional therapy in EE

Several clinical phenomena confirm an association between nutritional status and intestinal health and absorptive capacity. Exclusion of enteral nutrients in

otherwise normal humans (for example, small intestine anatomically out of continuity from the nutrient stream or in patients with anorexia nervosa (Winter et al., 2000)) results in villous atrophy and increased intestinal permeability; recovery with enteral feeding is enhanced with L-Glutamine and L-Arginine supplementation (Buchman et al., 1995). Enteral exclusion in normal humans also results in a reduction in brush border disaccharidase activity (Guedon et al., 1986). These abnormalities are also evident in severe acute malnutrition (Burman, 1965; Campos et al., 1979), although other pathophysiological processes including EE are likely to be contributing.

Nutrient deprivation alters TJ structure and function, and is associated with increased levels of pro-inflammatory IFNγ and TNFα and reduced levels of anti-inflammatory IL-10 in animal models (Demehri et al., 2016; Yang et al., 2009), as well as humans (Ralls et al., 2015). This results in increased small intestinal permeability.

Glutamine is the preferred fuel source for small intestinal enterocytes. Enteral glutamine supplementation improves gut permeability via an effect on tight junctions, inhibits mucosal atrophy, and improves intestinal permeability in animal models of physiological stress (Zhang and Jiang, 2015; Zou et al., 2010). These effects may be mediated through MTOR signalling (Boukhettala et al., 2012; Coëffier et al., 2013; Nakamura et al., 2012; Xi et al., 2012; Yi et al., 2015).

Furthermore, protein-energy malnutrition is associated with a variety of innate and acquired immunological abnormalities (Rytter et al., 2014) – most obviously in the poor rates of vaccine immunogenicity in stunted populations, and in the infectious mortality observed in severe acute malnutrition. The relative

contributions of EE and undernutrition to these phenomena have not been established and are likely to be interlinked. However there is increasing evidence from animal and *in vitro* models to support a direct effect of undernutrition on impaired immune function, with further evidence that correcting undernutrition can correct the immunological abnormalities (Iyer et al., 2012).

Several micronutrients have established roles in adaptive and acquired immunity, of which vitamins A and D are best studied (Mora et al., 2008; Ross, 2012). Vitamin A deficiencies in particular (even in the absence of clinically overt deficiency) is associated with increased mortality in the developing world. Other micronutrients including zinc and selenium have purported benefits on immune function, although their mechanisms of action are largely unknown.

1.4.3 Clinical trials of nutritional interventions in EE

1.4.3.1 Protein-energy supplementation trials

Most interventional trials for EE have been conducted in children, using lactulose : mannitol ratios and / or growth as a clinical endpoint. A number of studies in children have looked at protein-energy supplementation, with or without micronutrient supplementation and/or educational programmes (Adu-Afarwuah et al., 2007; Bhandari et al., 2001; Gartner et al., 2007; Kuusipalo et al., 2006; Lartey et al., 1999; Obatolu, 2003; Oelofse et al., 2003; Owino et al., 2007; Rivera et al., 2004; Roy et al., 2005; Santos et al., 2005; Schroeder et al., 2002). The interventions used are heterogeneous, and include milk-based, cereal/legume-based, or lipid-dense ready-to-use therapeutic food (RUTF)-based supplements.

These studies did not investigate intestinal permeability or other markers of the physiological abnormalities observed in stunted populations with EE. Robust systematic reviews have shown that optimising protein-energy intake has only modest effects on stunting of up to 0.7 HAZ scores (Bhutta et al., 2008; Dewey and Adu-Afarwuah, 2008).

1.4.3.2 Amino acid supplementation trials

A small number of studies have investigated the effect of glutamine supplementation on weight gain, growth and barrier function in EE in infants and children only. Infants with EE supplemented with L-Glutamine over a five month period did not show any improvements in growth or intestinal permeability (as measured by lactulose : mannitol ratio) when compared with an isonitrogenous, isocaloric mix of non-essential amino acids in Gambia infants (Williams et al., 2007).

An enteral glutamine supplement in hospitalised moderately to severely malnourished children resulted in improvements in intestinal permeability (as measured by lactulose : mannitol ratio) but did not show any benefit in terms of weight gain compared to an isonitrogenous control (Lima et al., 2005).

1.4.3.3 Lipid supplementation trials

Daily supplementation with n-3 long-chain polyunsaturated fatty acids (DHA and EPA) for 6 months improved some nutritional measures including MUAC and skin fold thickness, but did not improve growth, intestinal permeability (as measured

by lactulose : mannitol ratio), or cognitive development in Gambian infants, compared to olive oil placebo (van der Merwe et al., 2013).

1.4.3.4 Micronutrient supplementation trials

A two week course of zinc sulphate 20mg/day (equivalent to approximately 4.6mg of elemental zinc) given to one- to three-year-old children in rural Malawi protected against a deterioration in urinary lactulose : mannitol ratio which was observed in the placebo group (Ryan et al., 2014).

Multiple micronutrient supplementation appears to have subtle effects on the histological features of EE in urban Zambian adults (Kelly et al., 2010, 1999; Louis-Auguste et al., 2014). In rural Malawian children, MM supplementation results in a modest but significant improvement in lactulose : mannitol ratio (Smith et al., 2014).

1.5 Other interventions for environmental enteropathy

1.5.1 Antimicrobial interventions

A seven day course of Rifaximin, a non-absorbable semi-synthetic antibiotic, did not improve intestinal permeability as measured by lactulose : mannitol ratio in rural Malawian children (Trehan et al., 2009).

Helminth infection is common in impoverished communities, and may contribute directly and indirectly (through secondary effects on the bacterial microbiome) to intestinal dysfunction. A dose of albendazole given to 1-3 year old children in a

placebo controlled trial in rural Malawi protected against a deterioration in urinary lactulose : mannitol ratio after 34 days (Ryan et al., 2014).

Modification of the microbiome through the use of probiotics has also been trialled. A 30 day treatment with a daily supplement of 5^{10} *Lactobacillus* GG did not improve lactulose : mannitol or urinary sucrose : lactulose ratios (Galpin et al., 2005).

1.5.2 WASH interventions

The introduction of WASH interventions in a stunted population is expected to reduce the prevalence of stunting at three years of age by only 2.4%, although the incidence of diarrhoeal episodes might be reduced by 30% (Bhutta et al., 2008). More recently, robust clinical trials of WASH interventions have failed to demonstrate any benefit on linear growth (Luby et al., 2018; Null et al., 2018), although effects on intestinal permeability were not directly assessed.

1.5.3 Other interventions for EE

Mesalazine, a 5-aminosalicylic acid commonly used for the treatment of ulcerative colitis, has been used as an adjunctive treatment in severe acute malnutrition on a background of EE (Jones et al., 2014). Although there were some improvements in plasma inflammatory markers compared to placebo, there was no improvement in lactulose : mannitol ratio.

1.6 The mechanistic target of rapamycin complex 1 (MTORC1) cellular nutrient sensing pathway and its therapeutic potential in environmental enteropathy

The mechanistic (previously 'mammalian') target of rapamycin (MTOR) complexes are the most important global cellular nutrient and energy sensors, and function as a critical checkpoint in cellular differentiation, division, and metabolism (Efeyan et al., 2015; Laplante and Sabatini, 2012, 2009). Many of the abnormalities described above could be related to MTOR biology, which will now be reviewed.

1.6.1 MTOR and the MTOR complexes

MTOR is an evolutionarily highly conserved serine/threonine kinase which can form two different protein complexes (Table 1.4 and Figure 1.6). The MTOR complexes integrate growth, nutrient, and cytokine signals from the cellular milieu (Figure 1.7, p.52). MTORC1 and MTORC2 activation is an indication of the readiness and appropriateness for a cell to survive, grow, divide, or differentiate (Figure 1.8, p.52). Outside of cancer biology, MTORC1 signalling has been best studied in immune cells, and T lymphocytes in particular.

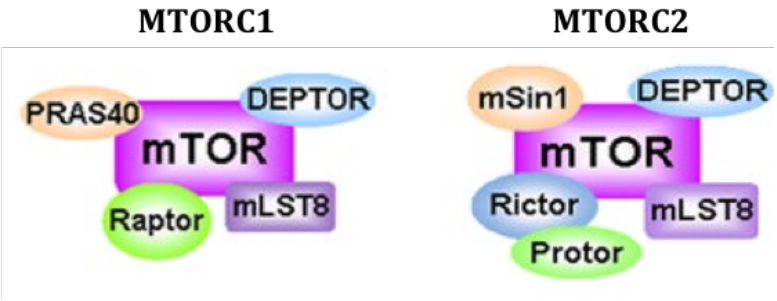

Figure 1.6. The MTOR complexes and their constituent proteins. Taken from Zhou and Huang, 2010.

Protein	Name	Complex	Function
MTOR	Mechanistic target of rapamycin	Both	Ser / Thr kinase; finally activated by disinhibition of TSC signalling
DEPTOR	DEP domain-containing MTOR-interacting protein	Both	MTOR inhibitor; degrades on MTOR activation
mLST8	Mammalian lethal with SEC13 protein 8	Both	Unknown
PRAS40	40 kDa Proline-rich AKT substrate	MTORC1	MTORC1 inhibitor; mediates AKT signalling
Raptor	Regulatory-associated protein of MTOR	MTORC1	Scaffold protein with regulatory roles; mediates AMPK signalling; binds activated RAG GTPase/Ragulator complex
mSin1	Mammalian stress-activated protein kinase-interacting protein 1	MTORC2	Scaffold protein
Rictor	Rapamycin-insensitive companion of MTOR	MTORC2	Scaffold protein, promotes AKT signalling
Protor	Protein observed with Rictor	MTORC2	MTORC2 kinase enhancer

Table 1.4. MTOR complex 1 and 2 constituent proteins and their functions.

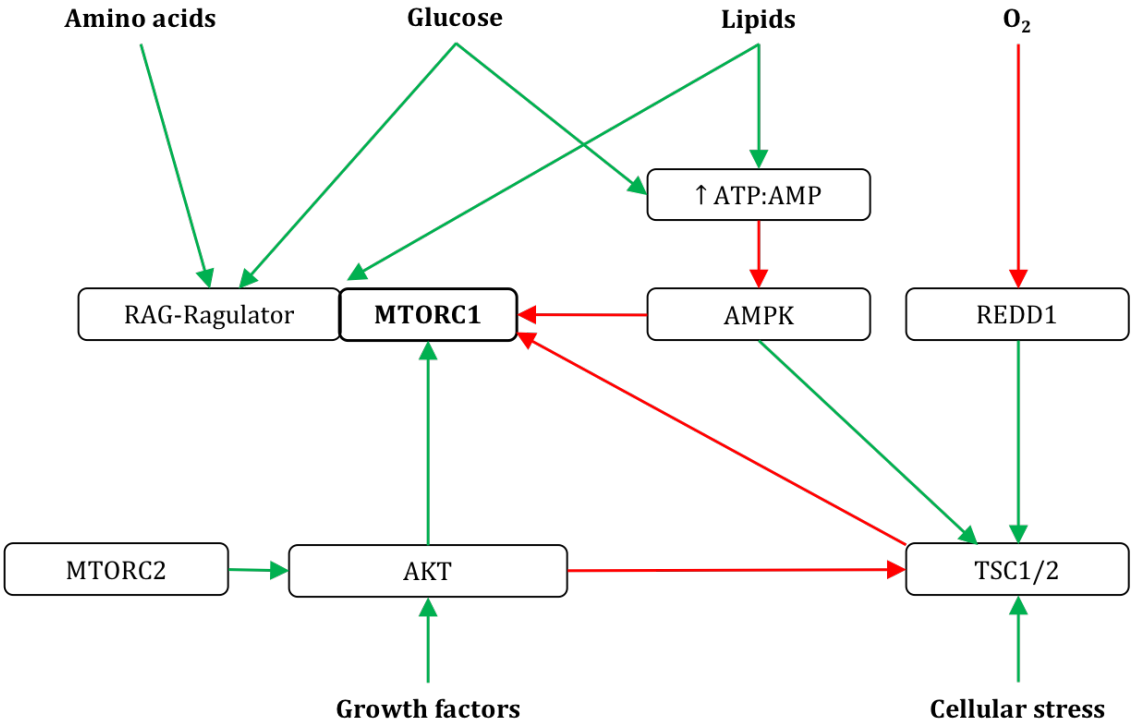

Figure 1.7. Overview of the integration of global cellular status signals by MTORC1. Green arrows represent activation; red arrows represent inhibition.

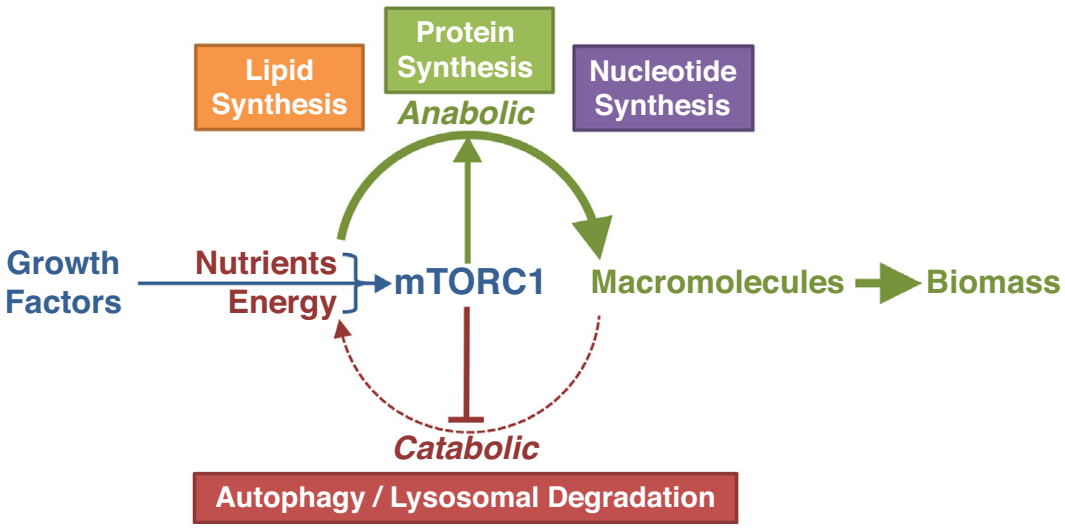

Figure 1.8. Overview of MTORC1 signalling. Taken from Ben-Sahra and Manning, 2017.

1.6.2 Cellular nutrient sensing by MTORC1

1.6.2.1 Amino acids

Amino acids are necessary for MTORC1 activity. Deficiencies of amino acids, and branched chain amino acids (BCAA) and essential amino acids (EAA) in particular, result in profound inhibition of MTORC1 activity (Avruch et al., 2009; Cobbold et al., 2009; Sancak et al., 2008). L-Leucine, a BCAA, is the canonical MTOR agonist (Hara et al., 1998).

The amino acid sensing mechanisms driving MTOR signalling are incompletely understood. Full MTORC1 activation occurs once the MTORC1 complex has been recruited to the lysosomal surface via the RAG GTPase-Ragulator complex (Sancak et al., 2008; Zoncu et al., 2011); Figure 1.9), which signals lysosomal (and thus intracellular) amino acid sufficiency detected by the amino acid transporter SLC38A9 (Rebsamen et al., 2015; S. Wang et al., 2015). This pathway is particularly responsive to lysosomal L-Glutamine, L-Leucine and L-Arginine levels, but other mechanisms exist, interacting with several aspects of the MTORC1 machinery. For example, there is also a RAG / Ragulator independent pathway for L-Glutamine sufficiency signalling (Jewell et al., 2015). Given that different upstream mechanisms are involved in AA sensing, it is unsurprising that the presence of different amino acids can have differential and synergistic effects on MTORC1 activation. For example, L-Glutamine supplementation has a synergistic effect on MTORC1 activity after EAA administration (Jewell et al., 2015).

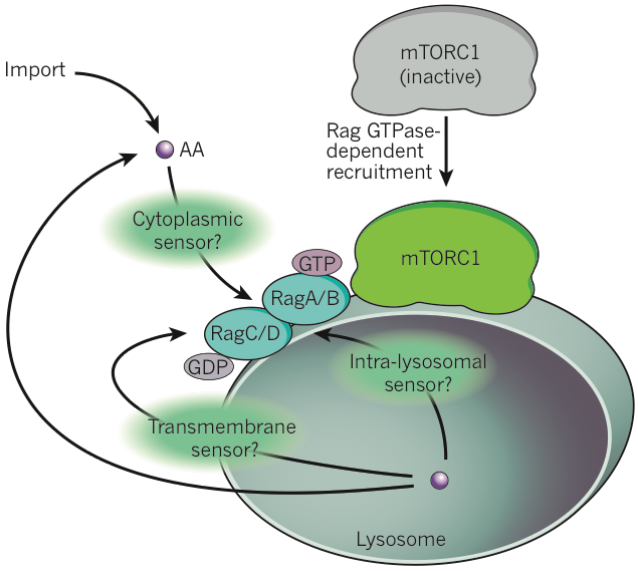

Figure 1.9. MTORC1 activation at the lysosomal surface requires amino acid sufficiency, which may be detected by numerous mechansims. Taken from Efeyan et al., 2015.

1.6.2.2 Glucose and global energy status

Glucose independently modulates the activity of the RAG GTPases through unknown mechanisms (Efeyan et al., 2013). Cellular carbohydrate levels are also sensed by glucokinase. This low affinity kinase converts glucose to glucose 6-phosphate which is subsequently consumed by glycolysis, resulting in increased levels of ATP and reduced levels ADP and AMP. The increased ATP:ADP and ATP:AMP ratio inhibits AMPK, which functions as a surrogate marker of glucose sufficiency (Efeyan et al., 2015). ATP:ADP and ATP:AMP ratios also reflect global cellular energy stores and are similarly measured by AMPK signalling. AMPK-induced phosphorylation in a low energy environment inhibits MTORC1 activity directly through the phosphorylation of Raptor and indirectly through the phosphorylation of the inhibitory tuberous sclerosis complex protein 2 (TSC2) (Gwinn et al., 2008; Inoki et al., 2003).

1.6.2.3 Oxygen

Hypoxia inhibits MTORC1 activity through the upstream induction of hypoxia-inducible factor (HIF) signalling via the regulatory protein REDD1. Under normoxia, low levels of REDD1 allow binding and inhibition of TSC2 by protein 14-3-3, which suppresses the inhibitory function of the TSC complex (DeYoung et al., 2008). In turn, MTORC1-mediated phosphorylation of HIF pathway proteins results in upregulation of glycolytic pathways which provides an energy burst in rapidly dividing activated immune cells, but may also drive tumorigenesis via the Warburg effect in mutated cells (Cheng et al., 2014; DeYoung et al., 2008; Finlay et al., 2012).

1.6.2.4 Lipids

Lipid sensing pathways remain incompletely understood, but feed in to the MTORC1 pathway indirectly via the global energy sensing function of AMPK. Lipids appear to regulate MTOR signalling primarily by promoting lysosomal recruitment and stability of the MTOR complexes. Phosphatidic acid (PA), produced either *de novo* for cell membrane synthesis or from the endogenous degradation of cell / organelle membranes, signals cellular sufficiency by directly stabilising the MTOR complexes (Foster, 2013; Foster et al., 2014), and promoting MTOR activation by binding the FKB12-Rapamycin binding domain (Fang et al., 2001; Toschi et al., 2009). Lysosomally-derived cholesterol activates the lysosomal amino acid transporter SLC38A9 independently of its amino acid sensing roles, promoting MTORC1 activation via activation of RAG GTPase complex, which binds the MTORC1 complex to the lysosome (Castellano et al., 2017).

1.6.2.5 Micronutrients

Several micronutrients appear to influence MTOR activity, either directly or indirectly. Extracellular zinc results in MTORC1/S6K1 activation through the PI3K/AKT pathway (Kim et al., 2000), and zinc chelation inhibits amino acid- and insulin-mediated phosphorylation of S6K1 via MTORC1 (Lynch et al., 2001), suggesting that it acts directly (at MTOR) and indirectly (via PI3K/AKT).

1,25-dihydroxycholecalciferol (vitamin D_3) promotes tolerogenic DCs *in vitro*, including antigen-specific tolerance (Ferreira et al., 2015). In healthy human monocyte-derived DCs, vitamin D_3 promotes tolerance via an MTORC1-dependent process which is associated with increases in both oxidative phosphorylation and oxidative glycolysis, although it appears that it is the glycolytic enhancement that is crucial for induction of tolerance (Ferreira et al., 2015). This is in contrast to the adaptive immune system, where induction of glycolysis by MTORC1 activation results in effector functions (discussed in section 1.6.6 below).

1.6.3 Other nutrient sensing pathways

The general control non-derepressible 2 (GCN2) pathway operates synergistically with the MTORC1 pathway, by signalling amino acid depletion (rather than amino acid sufficiency). GCN2 binds uncharged tRNA molecules, and does not discriminate between amino acid specificities (Dong et al., 2000). This allows GCN2 to respond to cellular deficiencies in any one amino acid (via its tRNA). Binding of uncharged tRNA activates GCN2, resulting in phosphorylation and activation of its target EIF2α (Zhang et al., 2002). This results in both a global downstream

inhibition of protein synthesis, and upregulation of catabolic enzyme pathways involved in the generation of essential amino acids (Zhang et al., 2002).

In parallel, the enterohumoral system senses ingested nutrients and secretes a variety of neurohumoral effectors. In addition to their canonical effects in regulating appetite, gastrointestinal motility, and metabolic signalling, many of these may also have secondary effects on immune function and gut trophism (Psichas et al., 2015). Glucagon-like peptide 2 (GLP2), for example, is secreted in the distal ileum in response to luminal amino acids delivery. GLP2 stimulates villus growth which increases small intestinal absorptive area and thus nutrient (including amino acid) absorption (Drozdowski, 2009).

1.6.4 Integration of signalling through MTORC1

Full activation of the MTOR complexes results in translocation from the cytosol to the lysosomal membrane. Recruitment of MTORC1 from the cytosol to the lysosomal membrane is mediated through the RAG GTPases-Ragulator complex which in turn is activated by AA abundance (Efeyan et al., 2015).

RHEB (Ras homolog enriched in brain) activation is the final step in activation of the MTORC1 complex. RHEB associates with MTORC1 at the lysosomal surface. RHEB is activated by a number of nutrient and energy signals including oxygen levels, ATP:AMP ratio, nutrient levels, WNT signalling, and growth factors such as insulin, mediated through the inhibitory TSC complex. RHEB phosphorylates and activates the catalytic domain of MTOR, resulting in activation of its kinase ability.

Phosphorylation of MTOR is differentially regulated, depending on whether it is part of MTORC1 or MTORC2. Full activation of the MTOR kinase requires phosphorylation at multiple sites and by multiple enzymes including RHEB, P70S6 (Chiang and Abraham, 2005; Holz and Blenis, 2005) (thus resulting in positive feedback), PI3K (Acosta-Jaquez et al., 2009), and auto-phosphorylation (Acosta-Jaquez et al., 2009) in an amino acid-dependent manner.

1.6.5 Downstream effects of MTORC1 activation

1.6.5.1 Activation of transcriptional and translational programs

Globally, MTORC1 signalling promotes anabolic signals and inhibits catabolic signals including autophagy. MTORC1 activation promotes cellular anabolic pathways, namely protein, lipid and nucleotide synthesis. This results in translation and protein synthesis, and a switch towards aerobic glycolysis to provide a rapid energy boost. Conversely, MTORC1 inhibition promotes fatty acid oxidation, prevents new transcription programs, and promotes autophagy (Figure 1.8, p.52 and Figure 1.10 below).

Activation of MTOR complex 1 (MTORC1) results in the phosphorylation of eurkaryotic initiation factor 4E-binding protein 1 (4EBP1) and ribosomal protein S6 kinase 1 (S6K1 or P70S6K1). Phosphorylated 4EBP1 dissociates from EIF4E, allowing it to form the EIF4F translation initiation complex. Phosphorylated S6K1 targets ribosomal P40S6 (rS6) among others, permitting translation (Figure 1.10). Activation of MTOR complex 2 (MTORC2) results in phosphorylation of AKT (also

known as protein kinase B), which among other functions promotes MTORC1 activity by phosphorylating and inhibiting TSC2 (Figure 1.7, p.52).

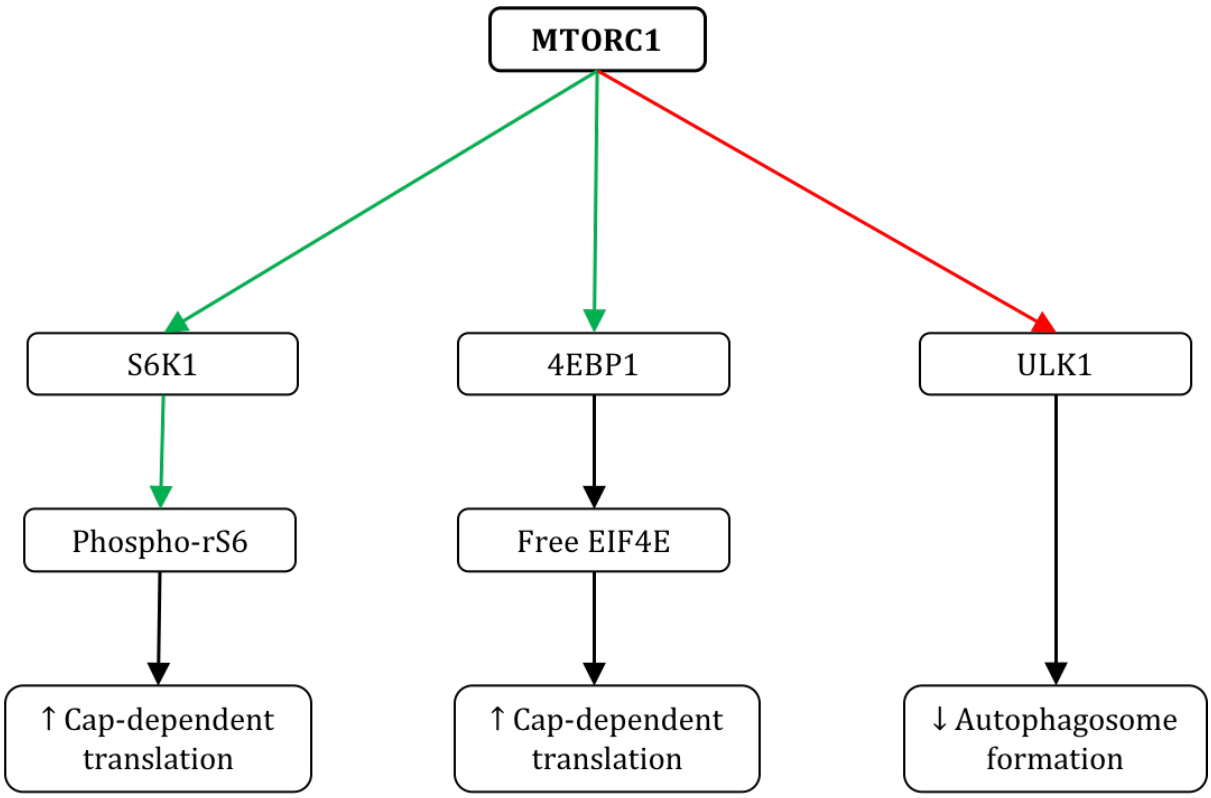

Figure 1.10. Downstream effects of MTORC1 activation. Green arrows represent phosphorylation and activation; red arrow represents phosphorylation and inhibition.

EIF4F (the end result of 4EBP1 phosphorylation) and S6K1 appear to be primarily involved in upregulating translation- and metabolism-related proteins. This occurs through a global, co-ordinated upregulation of a number of metabolic and synthetic programs involved in cell proliferation and metabolism. Functionally, MTOR-mediated (particularly MTORC1-mediated) translation promotes a number of metabolic and translational programs, including ribosomal biogenesis (Chauvin et al., 2014; Hsieh et al., 2012), post-translational modification (Hsieh et al., 2012),

biosynthesis (Düvel et al., 2010a; Hsieh et al., 2012), aerobic glycolysis (Düvel et al., 2010a), pentose phosphate pathway (Düvel et al., 2010a), and nucleotide synthesis (Ben-Sahra et al., 2016, 2013; Robitaille et al., 2013). Functionally and transcriptionally, 4EBP1 appears to be the most important mediator of MTORC1's downstream upregulated transcriptional program (Chauvin et al., 2014; Thoreen et al., 2012, 2009).

MTORC1-mediated phosphorylation of S6K1 and 4EBP1 is required but not sufficient for the initiation of cap-dependent translation. Activated MTORC1 phosphorylates the first of numerous 4EBP1 residues. Subsequent full phosphorylation, which is not fully MTORC1 dependent, results in the release of eukaryotic initiation factor 4E and cap-dependent translation (Gingras et al., 2001, 1999).

Similarly, rS6 is sequentially phosphorylated at multiple sites, with the initiation of phosphorylation undertaken exclusively by activated S6K1 (Ferrari et al., 1991; Pende et al., 2004; Roux et al., 2007). In turn, initial phosphorylation and activation is exclusively mediated by MTORC1 (Salmond et al., 2009).

1.6.5.2 Cell growth and cell cycle: transcriptional and translational control

The G1/S phase transition is the crucial transition point in rapidly replicating cells including enterocytes and activated immune cells (Massagué, 2004). These cells must respond to different external stimuli, for example growth factors for enterocytes and activation signals for immune cells. MTORC1 balances the 'request' for proliferation via the PI3K / AKT pathway against the 'readiness' of the

cell to divide in terms of nutrient availability. MTORC1 is one of the critical nodes that licenses cells to proceed through G1/S (Massagué, 2004).

1.6.5.3 Metabolic reprogramming

Although oxidative phosphorylation is far more efficient in terms of energy per unit of substrate (protein, carbohydrate or lipid) compared to glycolysis, glycolysis is able to provide a rapid burst of cellular energy which can be used by stimulated cells. MTORC1 supports this increased energy requirement by promoting aerobic glycolysis (the Warburg effect) by activating the downstream HIF1α pathway which results in upregulation of numerous glucose transporters as well as glycolytic enzymes (Düvel et al., 2010a; Finlay et al., 2012). Furthermore, MTORC1 inhibits lipolysis and β-oxidation of free fatty acids (Düvel et al., 2010a; Ricoult and Manning, 2013); by promoting lipogenesis this also has the secondary effect of providing an intracellular lipid store for cell membrane and organelle creation.

1.6.5.4 Autophagy

MTORC1 is a critical negative regulator of autophagy (Hosokawa et al., 2009; Kim et al., 2011). MTORC1 phosphorylates Unc 51-like kinase 1 (ULK1) and Autophagy Related Protein 13 (ATG13). Thus inactivated, the ULK1 complex is unable to initiate construction of the autophagosome. Unsurprisingly, AA deficiencies (and MTORC1 inhibition) promotes ULK1 activity (in conjunction with positive regulation from AMPK (Ben-Sahra and Manning, 2017; Kim et al., 2011)), resulting

in autophagy and recycling of cellular organelles, replenishing the cell's intracellular amino acid (as well as lipid, carbohydrate and nucleic acid) stores.

1.6.6 MTORC1 signalling and the immune response, with particular reference to CD4$^+$ T cell differentiation & function

MTORC1 signalling has been best studied in T lymphocyte (particularly CD4$^+$ T lymphocyte) differentiation and function. However, it is increasingly implicated in all branches of innate and adapted immunity (Table 1.5). In general, MTORC1 activity is required for a robust and effective activated immune cell response, for example in promoting aerobic glycolysis, the production of effector cytokine production, and rapid cell proliferation; conversely, cells stimulated in an environment resulting in MTORC1 inhibition tend to be anergic (e.g. impaired cytokine production; memory cell generation).

Cell type	Effect of MTORC1 activation	Effect of MTORC1 inhibition	References
CD4$^+$	T_{H1} T_{H2} T_{H17} generation	T_{reg} generation	(Waickman and Powell, 2012)
CD8$^+$	T_{eff} generation	T_{mem} generation	(Araki et al., 2009a)
B		Reduced plasma cell generation and antibody secretion	(Jones et al., 2016)
NK	Effector functions	Anergy	(Donnelly et al., 2014)
Dendritic cells	Activation	Anergy	(Krawczyk et al., 2010)
Monocytes	Trained immunity	Impaired survival and antigen response	(Cheng et al., 2014)
Innate lymphoid	Conflicting	Conflicting	(Weichhart et al., 2015)
NLRP3 inflammasome	Activation		(Moon et al., 2015)

Table 1.5. Summary of main effects of MTORC1 signalling in immune cells.

1.6.6.1 $CD4^+$ T cells

MTOR signalling has important roles in $CD4^+$ cell differentiation and homeostasis. T cells lacking MTOR are unable to differentiate into effector (T_{H1}, T_{H2} or T_{H17}) subtypes, and default to a T_{reg} phenotype (Delgoffe et al., 2009). MTORC1 signalling is required and sufficient for T_{H1} and T_{H17} differentiation (Delgoffe et al., 2011). Conversely, T_{H2} differentiation requires MTORC2 signalling, which does not affect T_{H1} / T_{H17} differentiation (Delgoffe et al., 2011). On the other hand, MTORC1 inhibition by EAA deprivation results in FOXP3 expression and T_{reg} differentiation; conversely T_{H1} / T_{H17} production results from EAA replete environments (Cobbold et al., 2009).

MTORC1 activation in undifferentiated T cells results in preferential differentiation to the T_{H1} immunophenotype through *TBET* transcription (Delgoffe et al., 2011) and then also phosphorylation and activation of the TBET transcription factor (Chornoguz et al., 2017). $TBET^+$ cells treated with Rapamycin fail to express IFNγ despite appropriate stimulation (Chornoguz et al., 2017). Similarly, Mtorc1 signalling is important for the maintenance of T_{regs} (Zeng et al., 2013).

As in other cell types, MTORC1 activity inhibits T cell autophagy (Li et al., 2006). MTORC1 activation results in a metabolic shift to aerobic glycolysis which supports effector T cell functions (Liu et al., 2014; Maciolek et al., 2014; Waickman and Powell, 2012).

1.6.6.2 MTORC1 inhibition: clinical effects and implications

Intriguingly, several studies have consistently demonstrated that MTOR inhibition in mice (and other eukaryotes) extends life by approximately 10% (Harrison et al., 2009; Johnson et al., 2013). Furthermore, nutrient (caloric) restriction also has a life-extending effect (Fontana et al., 2010; Johnson et al., 2013), and in fruit flies and rodents it appears to be essential amino acid (EAA) restriction that confers benefit (Mair et al., 2005; Miller et al., 2005; Orentreich et al., 1993). In mice at least, age-related changes in immune cell populations is influenced by the MTOR pathway, and inhibition is associated with longevity (Neff et al., 2013; Selman et al., 2009); indeed, the effect on longevity does not appear to be a general effect on ageing but is mediated through reductions in age-related morbidity / mortality in various organ systems including immunosenescence (Neff et al., 2013). The extent to which these findings can be extrapolated to humans is unknown.

A six week trial of MTOR inhibition with a rapamycin analogue (RAD001) in elderly volunteers resulted in significantly improved seroconversion rates and antibody titres to parenterally administered influenza immunisation, which was associated with a reduction in peripheral $CD4^+$ and $CD8^+$ lymphocytes expressing Programmed Death-1 (PD-1, an inhibitory and pro-apoptotic lymphocyte receptor upregulated in immunosenescence) (Mannick et al., 2014). However these results may not be applicable to the non-senescent mucosal immune system of EE, or to enterally administered vaccines.

The role of MTORC1 inhibition in the immune response to pathogens is largely unknown. However, there is some evidence that MTORC1 inhibition does result in a clinically relevant shift away from effector functions, as would be expected from

the *in vitro* data. In mice and non-human primates, inhibition of MTORC1 with rapamycin promotes and accelerates the generation of memory $CD8^+$ T cells in response to viral infection and vaccination (Araki et al., 2009b), and inhibits effector functions by preventing *TBET* expression (Rao et al., 2010). Similarly, pharmacological MTORC1 inhibition in a mouse DSS colitis model resulted in reduced T_{H1} and T_{H17} LP expansion and activation and promotes T_{reg} LP populations (Hu et al., 2016).

1.6.6.3 Potential role of MTORC1 signalling in T cell plasticity

Experienced immune cells are no longer viewed as being terminally differentiated. T cell plasticity describes the flexibility in immunophenotype and function observed in non-naïve T cells exposed to polarising conditions. Responding to mixed cues, naïve cells may differentiate into 'hybrid' cells, and experienced cells may lose their original function or gain additional function. In humans, significant plasticity of T_{H17} cells has been demonstrated (to / from T_{H1}, T_{H2}, and T_{reg}) (Brucklacher-Waldert et al., 2014; Cosmi et al., 2014; Sawant and Vignali, 2014; Vahedi et al., 2013). Cells with mixed phenotypes appear to have functions in between their parent types.

T_{reg} to T_{H1}-like transitions have been observed in mouse models (Hua et al., 2018) as well as humans PBMCs (Chowdary Venigalla et al., 2012). $TBET^+$ $FOXP3^+$ lamina propria lymphocytes are described in IBD, where they are correlated with the clinical severity of inflammation (Li et al., 2017). Indeed, loss of T_{reg} suppressor function and a switch towards a T_{H1} or T_{H17} phenotype has been observed in infection, and autoimmune conditions (Kitz et al., 2016). In humans, the

suppressor-to-T_{H1} phenotype is at least partly mediated through PI3K-AKT signalling (Kitz et al., 2016) and therefore the MTORC1 system. Constitutive MTORC1 stimulation, for example, will lead to loss of *FOXP3* expression and thus loss of the T_{reg} immunophenotype, even in the absence of inflammation (Sawant and Vignali, 2014).

Thus, modulation of MTORC1 signalling, in conjunction with other appropriate cellular signals, may be implicated in $CD4^+$ plasticity, although this has not been directly investigated.

1.6.7 MTORC1 signalling and the intestinal barrier

More recently, MTORC1 signalling has also been implicated in intestinal barrier and absorptive function. Its roles here have not been fully defined.

1.6.7.1 Ion exchange

Clinical use of rapamycin (sirolimus) is commonly associated with non-infectious watery diarrhoea (Joint Formulary Committee, 2018), and administration of rapamycin in mice and rats also results in a watery diarrhoea (Yang et al., 2015). This appears to be at least partly mediated through a down-regulation of sodium-hydrogen exchanger 3 (NHE3) expression on the ileal brush border, resulting in reduced Na^+/H^+ exchange (Yang et al., 2015). This is associated with both reduced levels of phospho-rS6 (indicating reduced MTORC1 and S6K1 activity) and phospho-AKT^{Ser473} (indicating reduced MTORC2 activity).

1.6.7.2 Epithelial cell growth and villous atrophy

Intestinal atrophy is commonly noted after non-enteral feeding, for example in post-surgical or ITU patients on total parenteral nutrition. Mice fed amino acid-free chow demonstrate mucosal atrophy (as assessed by a reduction in total mucosal height and villous density), which is associated with a reduction in enterocyte phospho-rS6 levels, reflecting reduced MTORC1 / S6K1 signalling. The protective effect of oral amino acid administration was negated by the parenteral administration of rapamycin (Nakamura et al., 2012). Furthermore, oral rapamycin can independently induce small intestinal mucosal atrophy in rabbits (Dias et al., 1998). MTORC1 activity in the normal fed state is most marked in the crypt (Nakamura et al., 2012). These findings are consistent with a role for amino acid-mediated MTORC1 signalling in intestinal stem cells in promoting mucosal regeneration.

L-Gln supplementation promotes growth in intestinal porcine epithelial cells (IPEC1) via MTORC1, independently of AMPK signalling (Yi et al., 2015). L-Arg supplementation similarly promotes cell growth and proliferation and protects against LPS-induced death via MTORC1 signalling in IPEC1 cells (Tan et al., 2010).

Dietary L-Arg supplementation in piglets improved small intestinal weight and villous height and reduced rates of IEC apoptosis, and this was associated with increased levels of MTORC1 activation (Wang et al., 2012).

1.6.7.3 Intestinal permeability and tight junction function

Rabbits administered oral rapamycin demonstrate an increase in transepithelial conductance and inulin flux (Dias et al., 1998), implicating MTORC1 signalling in the maintenance of TJ integrity. In a variety of *in vitro* models, MTORC1 signalling is implicated in maintaining TJ integrity. For example, butyrate in a mouse intestinal epithelial cell line protects against LPS-induced TJ permeability by upregulating MOTRC1 signalling which is associated with increased abundance of claudins 3 and 4 (Yan and Ajuwon, 2017). Exogenous zinc in Caco-2 cells increases transepithelial resistance and reduces TJ permeability through increasing signalling through the PI3K/AKT/MTORC1 pathway, which is associated with increased expression of the TJ protein ZO-1 (Shao et al., 2017). L-Trp supplementation in a porcine intestinal epithelial cells results in upregulation of a variety of TJ proteins associated with MTORC1 activation (H. Wang et al., 2015). L-Gln supplementation has similar physiological effects, and results in enhanced AMPK signalling (Wang et al., 2016) (with presumed downstream enhancement of MTORC1 signalling).

1.6.7.4 Nutrient uptake

Administration of rapamycin in rabbits results in increased active D-glucose uptake. Fructose uptake is unaffected (Dias et al., 1998). On the other hand, jejunal uptake of three of six fatty acids tested (18:0 [stearic], 18:2 [linoleic], and 18:3 [linolenic]) is reduced per unit of mucosa (Dias et al., 1998). Interestingly, only linolenic acid (18:3) ileal uptake was reduced with rapamycin, while 12:0 (lauric) and 18:2 (linoleic) uptake was increased (Dias et al., 1998).

Finally, MTORC1 stimulation upregulates the apical glucose transporter GLUT1 (Düvel et al., 2010b; Finlay et al., 2012).

1.6.7.5 Paneth cells, intestinal stem cells, and enterocytes

As well as having crucial roles in regulating and maintaining the intestinal stem cell niche and intestinal stem cell renewal (Clevers and Bevins, 2013), Paneth cells play an important role in regulating the microbiome and host defence (Bevins and Salzman, 2011b), partly through antimicrobial peptides (section 1.3.2.2, p.34).

Caloric restriction in mice results in increased intestinal stem cell proliferation and Paneth cell proliferation, but with reductions in mucosal / small intestinal mass and villous atrophy and with villi containing more immature enterocytes. These effects are mediated by caloric restriction-induced MTORC1 inhibition in Paneth cells (Yilmaz et al., 2012). The net effect is that in conditions of starvation, the intestine promotes self-renewal over villous growth and differentiation (Yilmaz et al., 2012). Furthermore, dietary restriction induces autophagy in Paneth cells (Hodin et al., 2011).

1.7 Summary

Environmental enteropathy is thought to underlie the phenomenon of stunting, a widely prevalent disorder in the developing world with lifelong detrimental consequences. EE is characterised by a number of intestinal and systemic physiological abnormalities, primarily pathologically increased intestinal

permeability and reduced small intestinal absorptive capacity on a background of a chronically inflamed gut. Although associated with undernutrition, sanitation and GI infections, interventional studies addressing these concerns have not resulted in improvements in stunting and / or the features of EE. Novel and practical therapies are therefore required. Furthermore, little is known about the relationship between the histological abnormalities, intestinal barrier function, and role of the mucosal immune system.

Populations affected by EE are prone to undernutrition. Nutrition has several important effects on the small intestinal barrier and mucosal immune compartment. Nutritional status, dietary intake, the intestinal epithelial barrier, the mucosal immune compartment, and the microbiota have complex and interdependent relationships that remain incompletely understood. There is laboratory and some clinical evidence supporting the role of micronutrients and / or amino acid supplements in maintaining intestinal health, and also some evidence of benefit in EE. Both amino acid and micronutrient deficiencies may be particularly relevant for intestinal immune and barrier function in individuals with nutritionally poor intake, and represent attractive targets for therapeutic intervention.

Global (protein-energy malnutrition; starvation; severe acute malnutrition) and specific (vitamin A and D deficiencies; glutamine deprivation) deficits have significant effects on absorptive capacity and the mucosal immune response. These effects are mediated through cellular nutrient sensing pathways. The MTORC1 pathway is the most important known cellular nutrient sensing mechanism, and is perhaps the most relevant for intestinal disorders due to its effects on both

immune cell and epithelial function. Nutrient, and in particular amino acid, sufficiency activates MTORC1 signalling. This encourages naïve $CD4^+$ T cells (and other immune cells) to differentiate into effector T cells, and promotes an effector-like immunophenotype in differentiated T cells. In the intestinal mucosa, MTORC1 signalling may have beneficial effects on intestinal permeability and epithelial growth. The role of MTORC1 signalling in the intestinal immune compartment, and its relationship to nutrient availability, is unknown.

1.8 Hypothesis, aims, and scope of this thesis

It was hypothesised that high dose multiple micronutrient supplementation and / or specific amino acid supplementation can modify some of the canonical features of EE (namely small intestinal morphometry and permeability), and that supplementation would concurrently modify lamina propria $CD4^+$ lymphocyte MTORC1 signalling (Figure 1.11). A phase II double blind randomised placebo-controlled trial was therefore conducted in Zambian adults with environmental enteropathy.

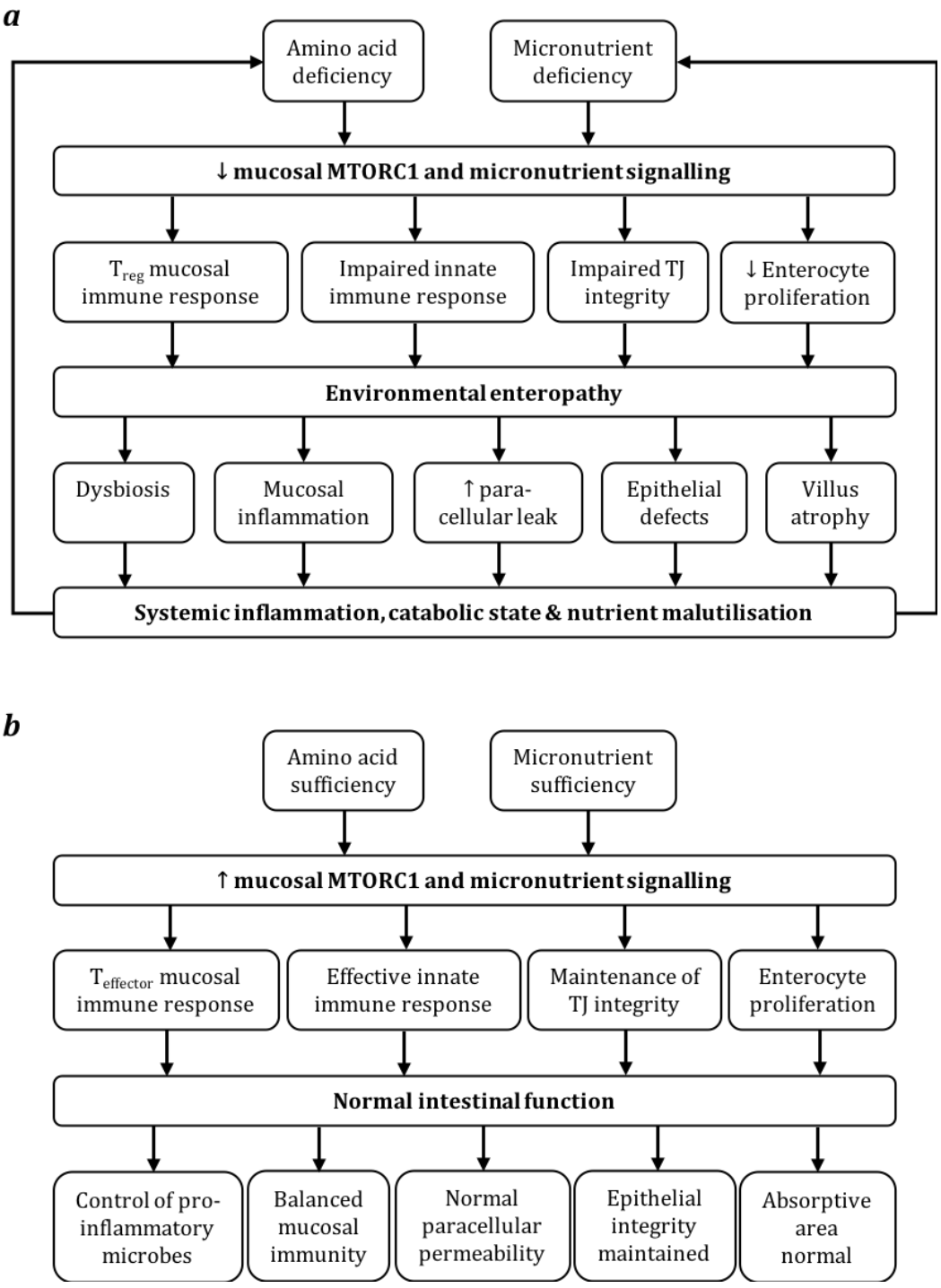

Figure 1.11. Hypothesis. a, Inter-relationship between nutrient availability, nutrient sensing through MTORC1, small intestinal barrier function and EE. b, Rationale for amino acid and / or micronutrient supplementation in EE.

1.8.1 Aims

The following aims are based on the primary and secondary outcomes and exploratory analyses from the trial (outlined in Chapter 2).

1.8.1.1 Aims (primary outcomes)

1. To assess the effect of supplementation on small intestinal histology as assessed by morphometry.
2. To assess the effect of supplementation on intestinal barrier dysfunction as assessed by confocal laser endomicroscopy.
3. To assess the effect of supplementation on lamina propria $CD4^+$ T cell MTORC1 signalling.

1.8.1.2 Aims (secondary outcomes)

1. To investigate the effect of supplementation on plasma markers of microbial translocation and immune activation (sCD14, CRP, LPS).
2. To investigate the effect of supplementation on metabonomic profile.
3. To investigate the effect of supplementation on anthropometry and body composition.

1.8.1.3 Exploratory analyses

1. To investigate the effect of supplementation on lamina propria $CD4^+$ MTORC1 signalling and TBET transcription.
2. To investigate the characteristics of the lamina propria immune compartment.
3. To investigate the effect of supplementation on plasma Glucagon-like peptide 2 levels.

1.8.2 Thesis outline

The bulk of this thesis describes the AMAZE trial (Amino acids and / or multiple Micronutrients in Adult Zambians with environmental Enteropathy).

Chapter 2 is an abbreviated version of the full trial protocol, and details how the trial was set up and undertaken.

Chapter 3 is the main methods chapter, and discusses the experimental methods chosen to study the intestinal barrier as well as describing the experimental procedures undertaken including preliminary / proof-of-concept work.

Chapter 4 provides the results of the trial's primary outcomes.

Chapter 5 provides the results of the trial's secondary outcomes and exploratory analyses.

Chapter 6 provides a general discussion.

CHAPTER 2

AMINO ACIDS AND / OR MULTIPLE MICRONUTRIENTS IN ADULT ZAMBIANS WITH ENVIRONMENTAL ENTEROPATHY (THE AMAZE STUDY): TRIAL OVERVIEW

2.1 Introduction

The effects of multiple micronutrient (MM) and / or amino acid (AA) supplementation in healthy adult volunteers with EE were assessed in a double blind, randomised, placebo-controlled trial using a 2×2 factorial design. Participants were randomised to receive MM supplementation or placebo, with or without an amino acid supplement of L-Glutamine, L-Leucine and L-Tryptophan or placebo, daily for 16 weeks. Participants underwent a number of clinical, laboratory and endoscopic assessments before and after the supplementation period to provide a global insight into the effects of the intervention on the various pathophysiological domains of EE. Outcomes were analysed according to HIV status, or together if there was no evidence of an effect of HIV.

The following paragraphs summarise the study protocol. The data collection instruments, patient information sheet and consent forms are included in Appendices 1-3 (p.205).

2.2 Study setting and participants

Healthy adult volunteers were recruited from an impoverished urban community on the outskirts of Lusaka, Zambia. The research clinic was located in this community, staffed by a full-time research nurse and two full-time clinical research assistants. Endoscopies, flow cytometry, ELISAs, and body composition analyses were performed at the University of Zambia School of Medicine in the University Teaching Hospital, Lusaka.

As in previous studies in this cohort, inclusion criteria were intentionally broad to allow truly representative sampling, and temporary exclusion criteria considered the possible confounding effects of recent infectious diarrhoea, antibiotic use and NSAID use.

2.2.1 Inclusion criteria

- Age between 18 and 60 years
- Resident in B section, Misisi compound
- Able to give informed consent

2.2.2 Exclusion criteria

- Pregnancy (by self-report)
- Breast feeding
- Antibiotic use within previous 4 weeks (defined as antibiotics taken regularly for more than 24 hours)
- Regular NSAID use within previous 4 weeks (defined as NSAID taken regularly for more than 72 hours)
- Diarrhoea within previous 4 weeks (defined as three or more watery stools for more than 24 hours)
- Significant comorbidity precluding endoscopy with sedation (at discretion of researcher)
- Therapeutic anticoagulation or bleeding diathesis precluding endoscopic biopsies
- Unwilling to undergo HIV testing
- Untreated helminth / *Giardia* infection, or helminth / *Giardia* infection treated within six months
- BMI < 18
- Unwilling to consent to long-term storage of samples

Participants were eligible to enrol in the study once any exclusion criteria had expired.

2.3 Recruitment and informed consent procedures

A robust three-stage system of screening, recruitment and consent was used which has been developed and tailored for use in the Misisi study population over many years: door-to-door notification, focus groups and individual face-to-face counselling leading to fully informed written consent. Following qualitative research in this population (Zulu et al., 2014), an interactive research facilities visit was also incorporated into the recruitment and consent process for this study.

Healthy adult participants were recruited from an impoverished community living in Misisi suburb in Lusaka, where previous work has demonstrated that EE is virtually universal (but present in varying degrees of severity). Experienced field workers conducted informal door-to-door discussions in B Section of Misisi compound to identify potential participants. Potential participants were told what the study involved in general terms, and that participation was voluntary.

Individuals who expressed an interest in participating at the time of door-to-door notification were invited to one of several focus group meetings. Individuals who had previously been involved in our clinical studies were also invited to attend.

The focus group discussions included the following:

- Introduction to the study team
- Introduction to the study
- Reading of the participant information sheet in Nyanja, which is universally spoken in this community. Literacy rates in Misisi are low and this was therefore the most appropriate method for conveying this information
- Benefits and risks of being involved in the research
- Previous participants' descriptions of being involved in research
- Questions to the study team and previous participants
- Invitation to attend a guided tour of the research and endoscopy facilities

Potential participants who were unable to make any of the focus group meetings were invited to the field clinic to run through the information sheet and discuss the study with a member of the study team.

Qualitative research undertaken in the Misisi community has identified that research participants have some misconceptions with regards to clinical research, and in particular what happens to the samples that participants provide (Zulu et al., 2014). The research facilities visit was designed to address these concerns in detail. Potential participants were invited to visit the research and endoscopy facilities. They were able to see the endoscopy unit, meet endoscopy staff, and were given demonstrations of the endoscopy equipment, in particular the taking of endoscopic biopsies. They were also shown the laboratory facilities and met the research staff, who gave explanations and demonstrations of what happens to samples once they have been taken. They were shown how small the biopsies and blood samples are. The handling and storage of biological specimens was also explained and demonstrated. Participants were invited to view researchers at work and observe experiments being conducted. These visits were informal and potential participants were encouraged to ask questions at any time.

Individuals who wanted to participate in the study were then invited to attend for written consent, screening and enrolment in the study. Consenting and enrolment occurred at least 48 hours after participants had attended a focus group meeting or study explanation visit. Participants were invited to ask further questions or express concerns. If they were happy to proceed, they were asked to sign or thumbprint the consent form, which was read out to them in Nyanja if they were unable to read.

2.3.1 HIV testing

All participants who were not known to be HIV positive were consented for HIV testing. HIV counselling and testing has been performed by the research clinic nurse both as part of standard medical care in the Misisi clinic and as part of the routine research process in our recent studies for over 15 years (Kelly et al., 2004, 2016).

HIV testing was performed using the UniGold point-of-care kit (Trinity Biotech Plc, Bray, Ireland). Positive results had a second confirmatory test performed using an alternative point-of-care kit. There were no cases where the result of the second

test was not confirmatory. New diagnoses were referred on to HIV specialists for further investigation and management including anti-retroviral therapy in accordance with World Health Organization and Government of Zambia Ministry of Health guidelines and procedures (Zambia Ministry of Health, 2014).

2.4 Study flowchart and schedule of assessments

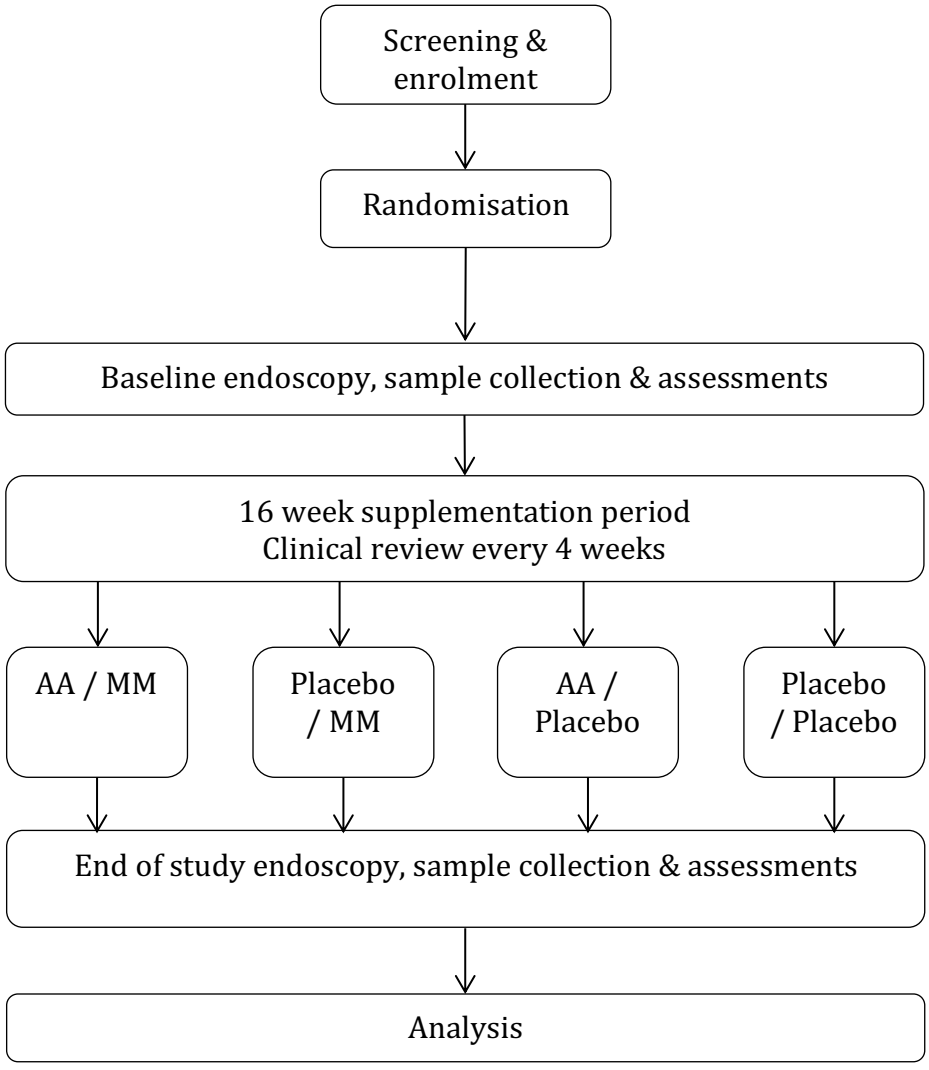

Figure 2.1. Study flow chart.

Visit number	1	2	3	4	5	6	7
Visit type	Screening / Enrolment	Pre-suppl. assessments	4 week visit	8 week visit	12 week visit	Post-suppl. assessments	End of study visit
Week	-4 to 0	0	4	8	12	16-19	16-20
History & physical examination	X						
HIV test	X						
Stool culture	X						
CLE & duodenal biopsies		X				X	
Blood, urine, saliva, stool samples		X				X	
Body composition & anthropometrics		X				X	
Compliance & AE monitoring			X	X	X		X
Supplement administration		X	X	X	X		

Table 2.1. Assessment and visit schedule. CLE, confocal laser endomicroscopy; AE, adverse event.

2.5 Randomisation and blinding procedures

Each amino acid / matched placebo sachet and each micronutrient / matched placebo pillbox was labelled with one of four alphanumeric characters (A, B, C, D for amino acid / placebo; 1, 2, 3, 4 for micronutrient / placebo). Two numbers and two letters were randomly assigned by the manufacturer to active supplement, with the other two therefore representing placebo. The supplements were then labelled accordingly by the manufacturer, and the allocation code supplied to the study statistician. The code was known to the manufacturer and to the study statistician, and was only revealed to the investigators once the databases had been locked.

After completing enrolment and baseline assessments, participants were randomly allocated to one of the 16 possible groups (4 possible AA allocations × 4 possible MM allocations) in blocks of 16 using a validated online randomisation service (www.sealedenvelope.com; accessed 10th September 2015). Participants were therefore allocated to each of the four study arms in a 1:1:1:1 ratio.

2.6 Study intervention

2.6.1 Design of supplements

2.6.1.1 Amino acid supplement and placebo

Considerations and choice of amino acids

L-Glutamine, L-Leucine and L-Tryptophan were selected for supplementation. Amino acid supplementation in EE is an attractive therapeutic target due to its potential effects on epithelial and lymphocyte MTORC1 signalling. Amino acids, particularly the BCAA L-Leucine and other EAA including L-Tryptophan appear to be the most effective activators of MTORC1 (Cobbold et al., 2009) and therefore are of particular interest for supplementation. L-Leucine is a particularly effective MTORC1 agonist (Cobbold et al., 2009).

L-Tryptophan is a precursor of niacin (vitamin B3). Niacin deficiencies result in pellagra ('dermatitis, diarrhoea, dementia') which is frequently observed in imporverished populations, and which causes intestinal inflammation via MTORC1 signalling in a rodent model (Hashimoto et al., 2012).

L-Tryptophan is partially metabolised by the inducible host enzyme indoleamine 2,3-dioxygenase (IDO) to produce kynurenines, which have anti-inflammatory effects and promote T_{reg} differentiation (McGaha et al., 2012; Murray, 2016; Yan et al., 2010). This pathway also results in L-Tryptophan deprivation and thus inhibition of MTORC1 (Metz et al., 2012). These host pathways may be subverted by microbial L-Tryptophan uptake (resulting in further host L-Tryptophan depletion and diversion away from host kynurenine production) and metabolism (resulting in pro- or anti-inflammatory metabolites which may include kynurenines) (Cervenka et al., 2017). In humans, low levels of L-Tryptophan and high levels of kynurenines are correlated with IBD activity (Nikolaus et al., 2017).

In stunted children with EE, plasma L-Tryptophan levels are correlated with growth, and declines with age (Kosek et al., 2016). Depending on the population studied, the kynurenine to tryptophan ratio (KTR, with high ratios indicative of high IDO activity) is moderately inversely correlated to growth: a high KTR and therefore greater degree L-Tryptophan shunting is associated with greater stunting (Kosek et al., 2016), suggesting that L-Tryptophan supplementation may be relevant.

L-Glutamine activates MTORC1 via at least two amino acid sensing mechanisms, and has synergistic effects when co-administered with EAAs (Jewell et al., 2015). L-Glutamine is also the preferential fuel for enterocytes (Rhoads and Wu, 2009; Windmueller and Spaeth, 1974). Studies of L-Glutamine supplementation in EE have not been conducted in adults, and have shown conflicting results in children (Lima et al., 2005; Williams et al., 2007).

Amino acid supplement dosage

Supplementation with the recommended nutritional intake (RNI) of L-Tryptophan and L-Leucine for a 70kg adult was considered to be an adequate dose. The RNI for

L-Tryptophan is 4mg/kg/day and the RNI for L-Leucine is 39mg/kg/day (Joint WHO/FAO/UNU Expert Consultation, 2007). The supplement therefore contained L-Tryptophan 280mg and L-Leucine 2.79g.

L-Glutamine is a non-essential amino acid and therefore does not have RNI values; however previous enteral L-Glutamine trials in EE or HIV enteropathy have used supplements varying from 16.2 – 30g/day or 0.25g/kg/day (Bushen et al., 2004; Leite et al., 2013; Lima et al., 2005; Williams et al., 2007), and enteral L-Glutamine supplementation trials in critical care have used doses up to 0.5g/kg/day (van Zanten et al., 2015). A dose at the higher end of those previously trialled was therefore selected (30g/day, or approximately 0.42g/kg/day).

The active supplement contained approximately the L-Tryptophan and L-Leucine content equivalent of 150g of beef (US Department of Agriculture, 2018). The dosages of all three amino acids are believed to be safe in long term use (Joint WHO/FAO/UNU Expert Consultation, 2007; Shao and Hathcock, 2008).

Choice of placebo

In order to avoid the possible confounding effect of an isonitrogenous placebo (used in previous L-Glutamine supplementation trials in EE (Lima et al., 2005; Williams et al., 2007)) on MTORC1 activity, an isocaloric placebo was used.

A perfectly taste matched placebo was not possible; however the slight bitter taste of L-Tryptophan was matched with the addition of a bitterant (Sodium hydrogen sulphite, E222) in the placebo.

Composition and presentation

The final composition of the amino acid supplement and placebo is given in Table 2.2. Both supplements were custom manufactured by Glanbia Nutritionals Deutschland GmbH, Orsingen-Nenzingen, Germany. The amino acid supplement and matched placebo were both colourless, odourless fine white powders, with a

mildly bitter taste. Active and placebo supplements were distributed in identical white foil packaging, printed with one of the four supplement codes.

	Active	Placebo
L-Glutamine	29.9g	–
L-Tryptophan	0.28g	–
L-Leucine	2.79g	–
Maltodextrin	–	32.9g
Sodium hydrogen sulphite (E222)	–	0.07g
Total	**33g**	**33g**

Table 2.2. Amino acid and matched placebo composition.

2.6.1.2 Multiple micronutrient supplement and placebo

Considerations

Individuals living in impoverished populations tend to have multiple micronutrient deficiencies, including our study population in Zambia (Gibson et al., 2011; Kelly et al., 2008). Because it is not practical to assess the effects of every micronutrient individually, micronutrient interventional trials tend to supplement with multiple micronutrients. Furthermore, multiple micronutrient supplementation is seen by many nutritionists as preferable to single nutrient supplementation as it more closely approximates a balanced intake.

Given the high prevalence of chronic deficiencies, high dose supplementation is preferable, both to replenish depleted stores and to maintain adequate physiological levels. Furthermore, previous work in this study population demonstrated that lower doses did not reliably increase the concentrations of the micronutrients in blood (Kelly et al., 2008), possibly reflecting the very malabsorptive problem which we are trying to overcome.

Although micronutrient supplementation is safe, iron (due to the risk of infectious complications (Sazawal et al., 2006)) and copper (due to theoretical concern of long-term toxicity) supplementation must be limited.

Available formulations

A high dose multiple micronutrient supplement was therefore selected. Many commercially available micronutrient supplements are available. The Immunace Original ® supplement (manufactured and donated by Vitabiotics Ltd., London, UK) was selected. This has low levels of iron and copper, and the retinoid doses in the supplement (5000iu) are known to be safe even in pregnancy (Rothman et al., 1995; WHO, 2004). Furthermore, no adverse events were recorded with this supplement in a previous study in this population (Louis-Auguste et al., 2014).

Placebo

An indistinguishable placebo was manufactured and donated by Vitabiotics Ltd., London, UK, consisting of only the excipients present in the active supplement.

Composition and presentation

The final composition of the micronutrient supplement and placebo is given in Table 2.3. The micronutrient supplement and placebo were indistinguishable tablets, and were packaged in identical plastic light-proof bottles of 60 tablets, labelled with one of the four supplement codes.

	Average dose per tablet	Total daily dose	EC RDA	Multiples of EC RDA per daily dose
Vitamin A RE (µg)	800	1600	800	2
Vitamin D3 (µg)	10	20	5	4
Vitamin E α-TE (mg)	40	80	12	6.66
Vitamin K (µg)	70	140	75	1.86
Vitamin C (mg)	150	300	80	3.76
Thiamin (mg)	18	36	1.1	32.72
Riboflavin (mg)	6	12	1.4	8.58
Niacin NE (mg)	27	54	16	3.38
Vitamin B6 (mg)	10	20	1.4	14.28
Folic acid (µg)	400	800	200	4
Vitamin B12 (µg)	14	28	2.5	11.2
Pantothenic acid (mg)	20	40	6	6.66
Iron (mg)	8	16	14	1.14
Magnesium (mg)	60	120	375	0.32
Zinc (mg)	15	30	10	3
Iodine (µg)	200	400	150	2.66
Copper (mg)	500	1000	1	1
Manganese (mg)	4	8	2	4
Selenium (µg)	180	360	55	6.54
Chromium (µg)	100	200	40	5
L-Cystine (mg)	40	80	N/A	N/A
L-Carnitine (mg)	30	60	N/A	N/A
Citrus Bioflavonoids (mg)	30	60	N/A	N/A
Excipients	Microcrystalline cellulose; hydroxypropylmethylcellulose; ethyl cellulose; propylene glycol; purified talc; titanium dioxide; iron oxides; glucose syrup; purified talc; magnesium stearate; silicon dioxide; polyvinylpolypyrrolidone; acacia; sucrose; starch; tri-calcium phosphate; dicalcium phosphate; medium chain tri-glycerides; colloidal silica; maltodextrin; butylated hydroxyanisole			

Table 2.3. Multiple micronutrient supplement composition. EC RDA, European Community Recommended Daily Allowance (European Union, 2008); RE, retinol equivalents; α-TE, alpha-tocopherol equivalents; NE, niacin equivalents. The micronutrient placebo contained only the excipients listed above.

2.6.2 Trial arms

Participants were randomised to receive multiple micronutrients or placebo and amino acids or placebo. The supplementation period was 16 weeks (112 days). Participants were instructed to take both supplements with their main meal every day, at any time of day, starting the day after the baseline endoscopy.

- **MM / placebo arm:** two Immunace ® Original capsules plus one "amino acid" placebo sachet, taken daily**.**
- **AA / placebo arm:** amino supplement plus two "micronutrient" placebo capsules, taken daily.
- **MM + AA arm:** two Immunace ® Original capsules and one amino acid sachet, taken daily.
- **Double placebo arm:** two "micronutrient" placebo capsules and one "amino acid" placebo sachet, taken daily.

2.6.3 Compliance monitoring

Participants were reviewed every four weeks by one of the research team, either at home or in the research clinic. Compliance was assessed by supplement count.

2.7 Endpoints

All outcomes were assessed by HIV status. The following trial primary and secondary outcomes in addition to a number of other exploratory analyses will be reported here.

2.7.1 Primary outcomes

(i) Change in small intestinal histology as assessed by morphometry according to treatment allocation.

(ii) Change in small intestinal barrier dysfunction as assessed by confocal laser endomicroscopy according to treatment allocation.

(iii) Change in lamina propria $CD4^+$ T cell MTORC1 signalling according to treatment allocation.

2.7.2 Secondary outcomes

(i) Change in plasma markers of microbial translocation and immune activation (sCD14, CRP, LPS) according to treatment allocation.

(ii) Change in metabonomic profile according to treatment allocation.

(iii) Change in anthropometry and body composition according to treatment allocation.

2.7.3 Other exploratory analyses

(i) Effect of supplementation on lamina propria $CD4^+$ MTORC1 signalling and TBET transcription.

(ii) Characteristics of the lamina propria immune compartment.

(iii) Effect of supplementation on plasma Glucagon-like peptide 2 levels.

2.8 Statistical considerations

2.8.1 Sample size and power calculation

The study followed a 2×2 factorial design and was powered to demonstrate the superiority of MM or AA over placebo in HIV negative participants, based on the findings of preliminary work which showed a potential benefit of MM supplementation in HIV negative individuals (Louis-Auguste et al., 2014). A 15%

difference in morphometry was expected to be associated with a similar degree of change in intestinal permeability, bacterial translocation and metabonomic profile. Changes of this magnitude were also expected to result in clinically meaningful differences in intestinal and immune function. One of the primary endpoints was therefore change in morphometry (villous height), and power calculations were based on morphometric data from a previous study of adults with enteropathy in Misisi (Louis-Auguste et al., 2014). These data were also similar to a previously published Misisi morphometric data (Kelly et al., 2004). Sample size calculations were powered for main effects and assumed no interaction between AA and MM effects.

To detect a difference of 15% between an intervention and the corresponding placebo required 56 HIV negative participants – 28 participants given the supplement and 28 given the corresponding placebo (expected difference in villous height 30μm; standard deviation 40μm; power 0.8; 2-sided α 0.05). Therefore 14 participants per intervention arm were required. Allowing for a 20% dropout rate and 30% HIV positive rate gave a total of 100 participants to be recruited.

2.8.2 Statistical analysis

As the primary outcomes were pathophysiological parameters in participants completing the study intervention, the primary analysis was per-protocol.

Due to relatively small sample sizes, non-parametric tests were preferred. Unless otherwise stated, values are given as median and interquartile range, the Mann-Whitney U test was used for independent samples, the Wilcoxon signed-rank was used for paired samples, and Spearman's *rho* was used for correlations. Parametric tests were used for some of the morphometry data, where the Shapiro-Wilk test confirmed normality.

Univariate 2-way ANOVA was used to examine for evidence of interaction between the supplementation arms, and data were examined for simple effects where an interaction was suggested.

Multivariate statistical modelling techniques were used to analyse metabonomic profiles, and were performed by Dr Jonathan Swann and Dr Jordi Mayneris, Department of Surgery and Cancer, Imperial College London.

2.9 Data collection

Clinical, demographic and endoscopic data was recorded on paper CRFs by a member of the study team at each study visit / procedure. Study data were double entered by trained personnel and managed using REDCap electronic data capture tools (Harris et al., 2009) hosted at Queen Mary University of London. REDCap (Research Electronic Data Capture) is a secure, web-based application designed to support data capture for research studies, providing: 1) an intuitive interface for validated data entry; 2) audit trails for tracking data manipulation and export procedures; 3) automated export procedures for seamless data downloads to common statistical packages; and 4) procedures for importing data from external sources.

A data check was performed at the end of the study for missing or implausible values. The database was locked and unblinded by the study statistician once the investigators were satisfied with the quality and integrity of the data.

2.9.1 Recording of adverse events

AEs and SAEs were recorded on appropriate case report forms throughout the study, and the severity of each adverse event was graded. The duration of the adverse event and postulated relationship to the study drug was also be recorded. If the AE was not defined as serious, the AE was recorded in the study file and the participant was followed up by the research team. The AE was documented in the participants' medical notes (where appropriate) and the CRF. SAE reporting was conducted in accordance with the Sponsor's and UNZABREC's requirements.

2.10 Regulatory aspects

2.10.1 Ethical approval

The study was carried out in accordance with the ethical principles in the Research Governance Framework for Health and Social Care, Second Edition, 2005 and its subsequent amendments as applicable and applicable legal and regulatory requirements. The study team followed the tripartite harmonised ICH guideline for good clinical practice E6(R1) 1996 with post-step 4 corrections.

The University of Zambia Biomedical Research Ethics Committee (UNZABREC; ref. 007-11-14) had statutory responsibility for overseeing study ethics. UNZABREC approved the final study protocol and all amendments, the case report forms, patient information leaflets, and consent forms. A further favourable non-binding opinion was obtained from Queen Mary University of London Ethics of Research Committee (QMERC; ref. QMERC2014/77).

2.10.2 Sponsorship

Study sponsorship was provided by Queen Mary University of London (ReDA 010299).

2.10.3 Regulatory approvals: Zambia

The legislation regulating human biomedical research in Zambia is enacted through the Health Research Act (2013). The following required approvals were obtained:

1. Zambian Medicines Regulatory Authority (ZAMRA; ref. CT054)
2. National Health Research Authority (Zambia; ref. MH/101/23/10/1)
3. Material transfer agreement

2.10.4 Regulatory approvals: UK

The Medicines and Healthcare Products Regulatory Authority (MHRA) provided an opinion that the trial was regarded as 'non-CTIMP' (Clinical Trial of an Investigational Medicinal Product) for the purposes of the Sponsor.

Storage of human specimens in the UK was approved by the Barts Health Human Tissue Resource Centre on behalf of the Human Tissue Authority, in accordance with the Human Tissue Act 2004.

2.10.5 Trial registration

The trial was registered with the Pan-African Clinical Trials Registry (http://www.pactr.org/), registration number PACTR201505001104412.

2.11 Specific ethical considerations

2.11.1 Consent

The study population has high levels of illiteracy and low levels of formal education. The 3-stage system of consent which was used (section 2.3, p. 77) which permits valid informed consent. This process has evolved over many years and has been tailored for use in this population. Recent qualitative research in this community has also suggested ways of improving the informed consent process further which were incorporated into this study, such as offering guided visits to the research facilities (Zulu et al., 2014).

2.11.2 Supplements

The benefits of these therapies in EE are potentially great but currently unknown, which justified their use in this study. A commercially available high-dose multiple micronutrient supplement was used which was well tolerated in a previous study

in this population without any serious adverse events (Louis-Auguste et al., 2014). The micronutrient supplement and the amino acid supplement are believed to be safe even in long term administration.

2.11.3 Endoscopies

Participants underwent two diagnostic endoscopies for research purposes only. Endoscopy in this population sometimes reveals potentially serious but treatable pathology (such as peptic ulcers or varices) which would otherwise have gone undiagnosed, and therefore may provide a direct benefit. Abnormal pathology identified at endoscopy was investigated and managed and / or referred on appropriately and free of charge for the duration of the study.

The risk to participants from the endoscopy is extremely low, and was performed under conscious sedation to minimise discomfort.

2.11.4 HIV testing

HIV infection critically affects the mucosal immune compartment. All participants were therefore required to consent to HIV testing to enrol in the study, and a number of participants were newly identified with this disease. Effective anti-retroviral treatment is now freely available in Zambia, and early diagnosis is therefore beneficial from both the individual and population perspective.

Consenting for HIV testing and communication of results was performed by an experienced and fully trained team of research staff and counsellors. HIV counselling and testing has been performed by this team both as part of standard medical care in the Misisi clinic and as part of the routine research process for several years.

HIV test results were given in person and in confidence at the community research clinic. With their consent, newly diagnosed patients were referred on to HIV specialists for further investigation and management including anti-retroviral

therapy, in accordance with WHO / Government of Zambia Ministry of Health guidelines and procedures (Zambia Ministry of Health, 2014).

2.11.5 Compensation for participation

As in our previous Misisi studies, participants and their immediate families were offered healthcare free of charge during working hours based at the research clinic in Misisi for the duration of the study. Medication, diagnostic tests, and costs related to inpatient assessment / treatment during this time, regardless of cause, were also provided free of charge if required.

Misisi participants were compensated 80 ZMW (approx. 8 GBP or 12 USD in 2015) for each of the hospital visits required for endoscopy and sample collection, which is an appropriate local level of reimbursement. Transport to / from the endoscopy unit and lunch was provided free of charge.

2.11.6 Local / cultural issues

The research team has been conducting clinical research in this population cohort for over twenty years. This has only been possible through establishing an excellent relationship with the Misisi community at the research clinic, and with experienced and dedicated local staff.

Qualitative work has shown that previous participants' experience of being involved in research is positive, that the community trusts the study team, and that they would consider being involved in further research projects (Zulu et al., 2014). It has highlighted that major reasons for volunteering include possible future medical benefits to the community arising from the research and access to healthcare. The major reasons for not wanting to participate have been concerns / misconceptions regarding the amount, handling, storage and use of samples taken. Peer advice not to participate is usually based on these misconceptions. Importantly, participating in a laboratory visit addressed their concerns and improved volunteers' perceptions (Zulu et al., 2014).

2.11.7 Supplement sharing

Supplement sharing is a well documented issue in interventional trials in the developing world. In order to limit this, during the focus group meetings, face-to-face consultations, and subsequent study visits it was emphasised to participants that:

- the supplement was of unknown benefit
- there was an equal chance of receiving inactive placebo
- the best chance of benefit was by taking the full supplement dose as prescribed
- the safety of the supplements has not been assessed in children
- the supplements were designed for adult use only
- each participant had been individually assessed to ensure that supplementation was suitable for him / her
- the supplements were not for sharing

2.11.8 Use of a placebo arm

Because there are currently very limited data on the effects of micronutrient or amino acid supplementation in EE, it was ethical to have a group given placebo treatments for several weeks as the condition of equipoise was fully met. Furthermore, participants receiving placebo were not disadvantaged by being involved in the trial.

CHAPTER 3

METHODS

3.1 Overview of available techniques for studying the intestinal barrier in environmental enteropathy

3.1.1 Light microscopy and morphometry

The simplest technique for studying the epithelial barrier is by light microscopy of endoscopic intestinal biopsies. In particular, well orientated endoscopic biopsy sections can be used to measure objectively a variety of morphometric parameters. These objective two dimensional measurements provide an assessment of three dimensional features including villous surface area.

3.1.2 Confocal laser endomicroscopy

Confocal laser endomicroscopy (CLE) is a relatively new endoscopic technique which allows *in vivo* confocal microscopic images of the intestinal mucosa to be obtained at the time of endoscopy, using intravenous fluorescein as a fluorescent contrast agent. Individual cells and microscopic defects in the epithelium can be readily distinguished, as well as goblet cells and IEL. Structures including subepithelial capillary networks can also be identified.

3.1.3 Immunohistochemistry

Commercially available antibodies to various tight junction proteins and different epithelial cell types exist, and can be used to visualise cellular distribution, protein expression and subcellular localisation of these proteins using standard immunohistochemistry techniques.

3.1.4 Advanced microscopy

Scanning and transmission electron microscopy has been used to demonstrate ultrastructural detail of the intestinal barrier including by using endoscopic biopsies, but has not previously been described in EE.

3.1.5 Urinary sugar recovery

These tests provide a safe and non-invasive measurement of intestinal permeability and absorptive function, and as such are suited to studies of enteropathy in children in particular. Sugar tests have therefore become widely used in studies of enteropathy. Studies of adults and children with EE typically show reduced urinary recovery of absorbable sugars such as xylose (indicating impaired absorptive capacity) and increased urinary levels of inabsorbable sugars such as lactulose (indicating increased intestinal permeability).

The assay involves administering a solution containing known quantities of absorbed and non-absorbed sugars. All urine over the following 3-5 hours is collected and the percentage of sugar excreted is calculated from the known urinary volumes and urinary sugar concentrations.

Results are usually expressed as the ratio of non-absorbable-to-absorbable urinary recovery percentage (such as lactulose to xylose). Normally, this ratio will be low; conversely in the highly enteropathic bowel, significant amounts of non-absorbable sugars will be taken up through the permeable gut barrier, and reduced amount of absorbable sugars will be absorbed due to a reduced absorptive surface area.

3.1.6 Other biomarkers of increased intestinal permeability

Non-excreted plasma proteins are normally excluded from the intestinal lumen, but may leak into the gut lumen and become detectable when there is pathological permeability. Of these, α1-antitrypsin levels (α1AT) ELISA is well established.

3.1.7 Other absorption studies

Small intestinal selenium and zinc absorption can be assessed by measuring plasma selenium and zinc levels before and after administration of a test solution. Zinc and selenium physiology is complex. Zinc is absorbed in the proximal small bowel, and secreted in the distal small bowel. Thus plasma zinc levels reflect the balance between these two processes.

3.1.8 Plasma biomarkers of microbial translocation and immune activation

Plasma levels of microbial translocation and immune activation markers can be quantified by ELISA. Furthermore, plasma LPS levels can be quantitatively assayed using the *Limulus* amoebocyte assay, a colorimetric technique which has been widely taken up as the standard for LPS detection in pharmaceutical and biomedical applications.

3.1.9 Summary: choice of methods used

The methods chosen were selected due to local experience, resource limitations, and logistical support. Obtaining small intestinal tissue is a unique resource in this field, and formalin fixed / paraffin embedded tissue has the additional advantage of allowing further histological sections and staining to be done in the future if needed (including immunohistochemistry, which at the time of the study was not readily available at the research facility in Zambia), and of being readily and safely exportable for analysis in other facilities.

CLE is resource intensive, but similarly the output (confocal images) can be digitally recorded and stored for analysis at a future time. A CLE system was recently installed in the research unit in Zambia and was available for use.

Measuring plasma levels of antigens of interest by ELISA is feasible in Zambia. A biomarker panel consisting of plasma LPS, CRP and sCD14 was chosen to provide assessments of bacterial translocation and systemic immune activation.

Urinary sugar recovery assays are well established, but facilities for analysis do not exist in Zambia and therefore requires exporting of the samples on dry ice. Although samples were taken, unfortunately due to unforeseen problems with equipment failure in the UK it was not possible to analyse urinary sugar recovery in the timeframe of this work, although this is planned for the future.

Measurement of plasma levels of micronutrient metals using optical emission spectrophotometry was planned using available research facilities in Zambia. Although samples were taken, it was not possible to analyse these in the timeframe of this work due to equipment problems, although again this is planned for the future.

3.2 Morphometric analysis of endoscopic small intestinal biopsies

3.2.1 Principle

Small intestinal absorptive area (a two dimensional surface covering a three dimensional structure) can be estimated in various ways. In recent years a number of potentially promising optical techniques such as optical coherence tomography are being developed, but as yet none of these novel techniques are sufficiently developed or validated to be used *in vivo* for examination of the small bowel.

Direct assessment of small intestinal structure has traditionally been assessed by morphometry, which measures various defined parameters in small intestinal architecture. This established technique has the disadvantage that it requires invasive tests (endoscopy) to obtain tissue samples, and is time consuming.

Morphometry allows the direct assessment of a number of structural parameters. Histological sections provide a two-dimensional cross section of a three-

dimensional structure, such that 2D cross-sectional villus area reflects 3D villus volume, and 2D villus perimeter reflects 3D villus surface area.

Enteropathies generally demonstrate variable and patchy microscopic changes; however, previous work in this study population has demonstrated that intra-subject variance is low (i.e. the enteropathic abnormalities observed in EE are stable when samples taken from different parts of the small bowel are compared in any given individual) (Kelly et al., 2004).

3.2.2 Method

Standard endoscopic biopsies were taken from the distal duodenum at the end of the endoscopy procedure. 2-3 biopsies were orientated on acetate strips under the dissecting microscope and fixed in 4% formaldehyde immediately post-procedure and subsequently paraffin embedded. 4 μm sections were stained with haematoxylin & eosin as per standard protocols. Embedding, sectioning, and H&E staining was performed by Mr Isaac Mweemba, Department of Histopathology, University Teaching Hospital, Zambia.

Stained sections were first assessed for adequacy of orientation by a single investigator blinded to treatment allocation (JL-A), where both crypts and villi were seen in longitudinal section (Figure 3.1). Adequately orientated sections were then digitised using a NanoZoomer S210 digital slide scanner (Hamamatsu Photonics UK Ltd, Welwyn Garden City, UK). Sections were reviewed using NDP.view2 v2.3.11 (Hamamatsu Photonics KK, Hamamatsu, Japan). Areas of sections suitable for analysis were exported as TIFF files at 100X magnification.

Images were analysed using a custom macro written in ImageJ (Appendix 4, p.219). Briefly, the image was thresholded and the villus-crypt interface identified visually. A villus bounding box was automatically drawn, measuring maximal villus height (VH) and maximal perpendicular villus width (VW). Cross sectional villus area (VA, as a measure of villus compartment volume) and villus perimeter (VP, as a measure of epithelial surface area) were automatically measured. Lines measuring crypt depth (CD) and muscularis mucosae underlying the villi of interest were drawn by eye and automatically measured (Figure 3.2). Recorded

parameters were mean VH, VW and CD, and VP and VA per unit length of muscularis mucosae (arbitrarily set at 100µm).

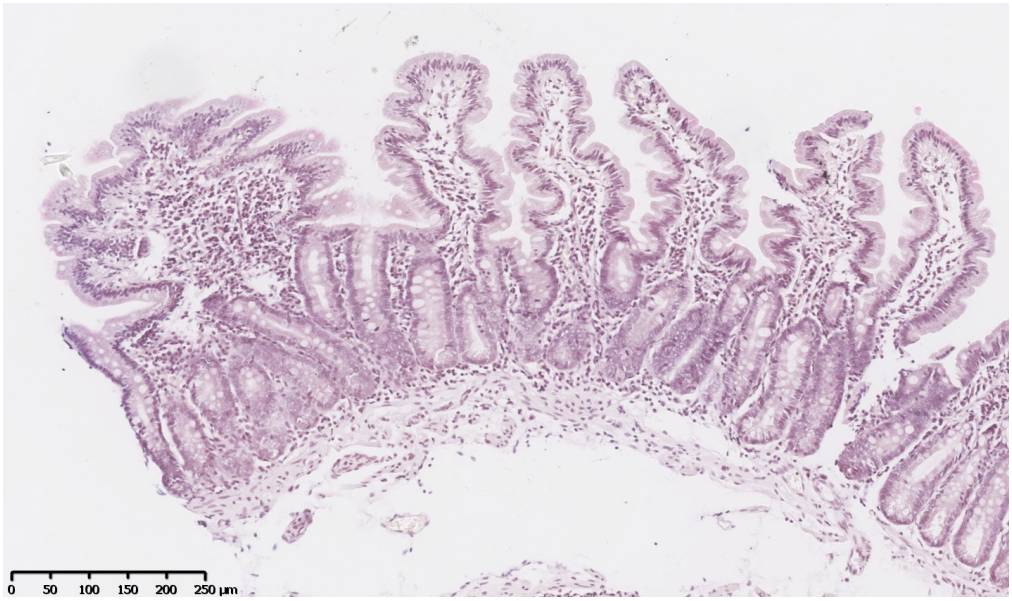

Figure 3.1. Example of adequately orientated section. This specimen also demonstrates moderate enteropathy, as evidenced by reduced villous height to crypt depth ration (<1.5:1), lymphocytosis, and a fused villus on the left.

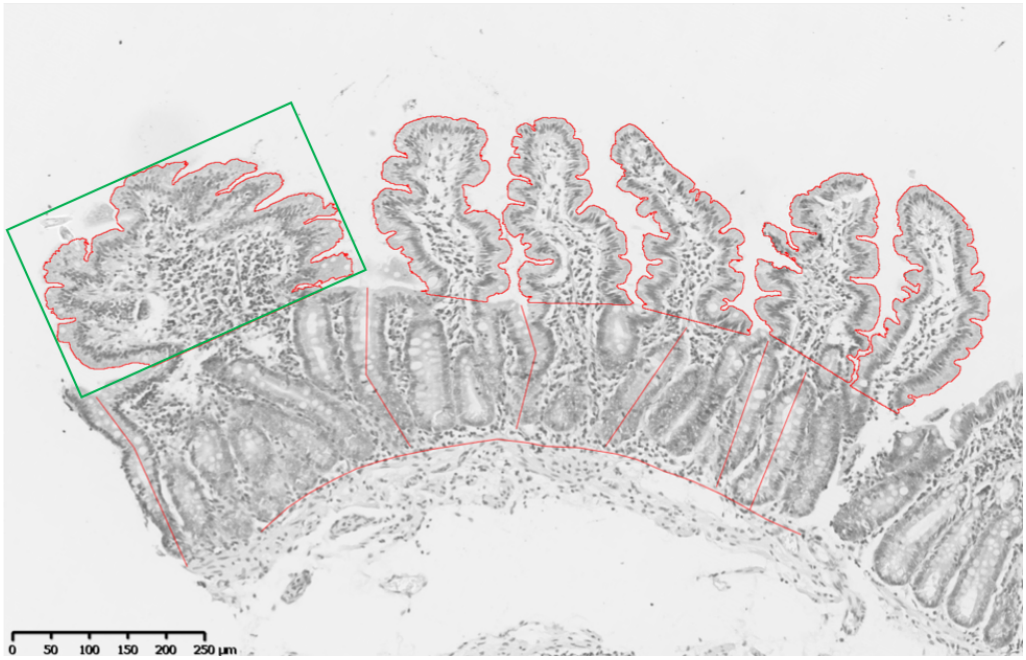

Figure 3.2. Example of computer-aided morphometry. An automatically generated villus bounding box (to measure VH and VW) is shown in green. VP was automatically measured from thresholded villi (outlined in red). Lines representing crypt depth and length of underlying mucosa were drawn by eye.

3.3 Confocal laser endomicroscopic assessment of small intestinal dysfunction in EE

3.3.1 Introduction

The canonical features of EE include increased permeability, mucosal inflammation, and villous atrophy. Confocal laser endomicroscopy (CLE) is a relatively novel technique which potentially allows the assessment of these features *in vivo* and in real time. Although originally envisaged as an 'optical biopsy' method for the detection and assessment of neoplasia *in vivo*, more recently a number of investigators have used the technique to assess non-neoplastic small intestinal dysfunction.

CLE scoring systems have been devised for inflammatory disorders of the small bowel including inflammatory bowel disease and coeliac disease. As many of the pathological features of EE are shared with these conditions, elements of these scoring systems may be applicable to the study of EE.

3.3.2 CLE priniciples

The principle of confocal microscopy is described in detail elsewhere (Tovey et al., 2006). Confocal microscopy allows greater vertical and horizontal spatial resolution than traditional wide field microscopy including fluorescence microscopy. In summary, high intensity coherent light from a laser source is focussed on the desired focal plane through a pinhole aperture. Emitted light from excited fluorophores on the target specimen passes back through a pinhole aperture which excludes out-of-focus light arising from surrounding areas (i.e. non-confocal light). Light reaching the detector through this confocal aperture is therefore in sharp focus, but collects in-focus light from a single point at a single depth. In order to create a 2-dimensional image of the target sample, the focal point scans across the target sample, allowing a 2-dimensional image to be reconstructed (Figure 3.3).

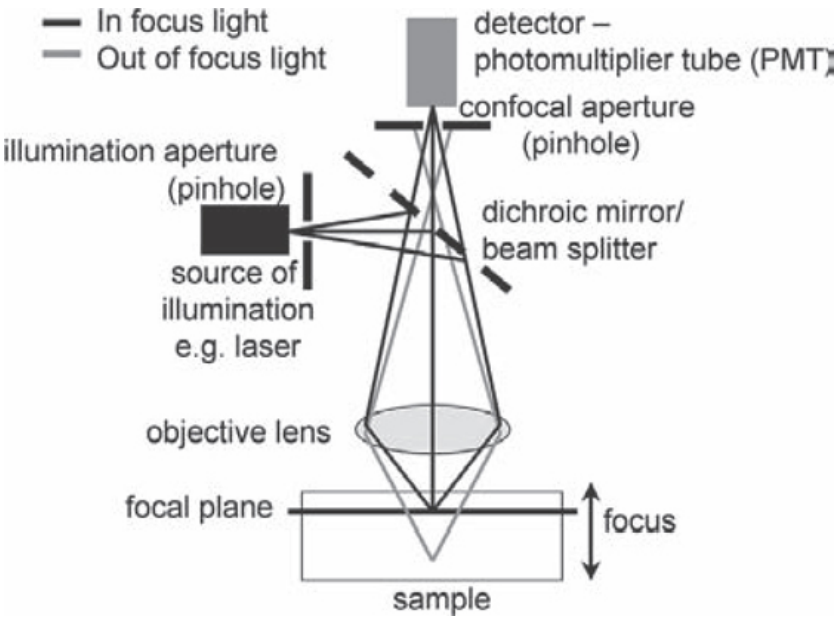

Figure 3.3. Confocal microscopy principle. Taken from Tovey et al., 2006.

In the confocal laser endomicroscope, the scanning laser light source and confocal aperture are integrated into the endoscope, with 2-dimensional images reconstructed in an image processing unit in the confocal stack. The Pentax CLE-enabled gastroscope (Pentax EC-3870CLK, Pentax Corporation, Tokyo, Japan; Figure 3.4) combines a supra-high definition CMOS-based digital white light gastroscope with an integrated 488nm laser and confocal detection system. The sensor detects fluorescent light at 505-585nm. Maximum resolution is 1024×1024 pixels at up to 1.6 frames per second, equivalent to a field of view of 475×475μm, with a maximum lateral resolution of approximately 0.7μm. Depth of scanning (*Z* axis) and imaging can be adjusted by the user between 0 and 250μm at 4μm intervals.

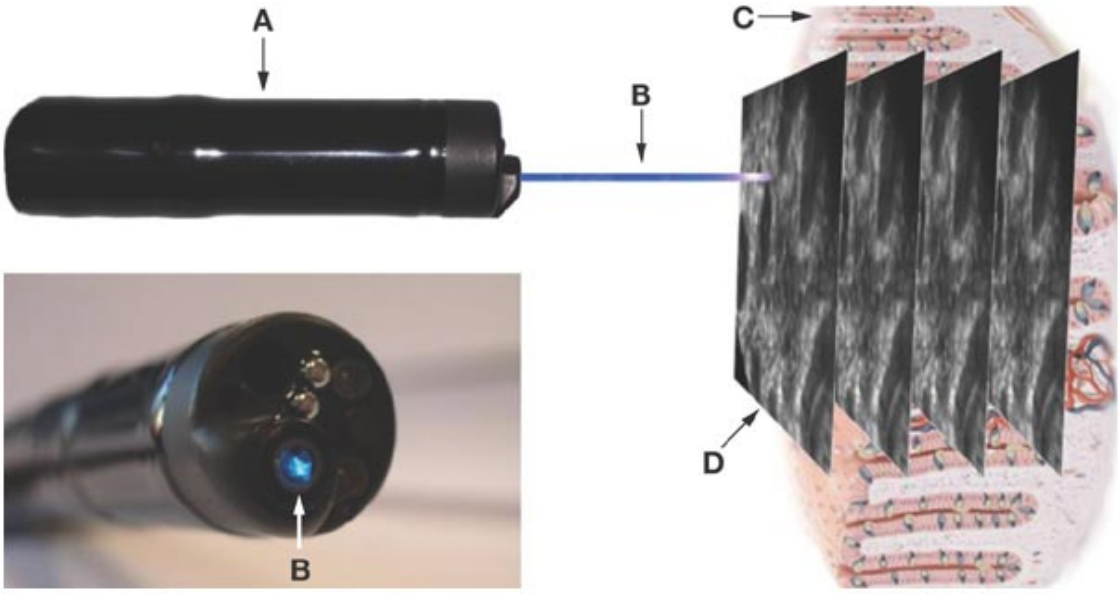

Figure 3.4. The Pentax integrated CLE system. A, distal end of the Pentax EC-3870CLK confocal laser endomicroscope. B, an integrated laser produces coherent light at 488nm which can be scanned across target tissue C at the desired focal depth. Emitted fluorescence passes through the integrated confocal aperture in the endoscope tip. By scanning across target tissue, 2-dimensional images can be reconstructed in various user-controlled planes (D). Taken from Kiesslich et al., 2007.

3.3.3 Published scoring systems to assess the epithelial barrier and intestinal inflammation using CLE

3.3.3.1 Inflammatory bowel disease

Watson score (Kiesslich et al., 2012)

This is a prospectively validated score which successfully predicted clinical relapse in inflammatory bowel disease, and remains the only published prospectively validated scoring system of intestinal barrier dysfunction using CLE. It is calculated by combining two assessments (Table 3.1):

1. Cell shedding events (equivalent to epithelial defects, either single shed cells or microerosions, defined as an exposed lamina propria with multiple shed cells)
2. Local barrier dysfunction, characterised luminal fluorescein leak (defined as fluorescein plumes, or luminal fluorescein rendering the lumen as bright or brighter than the epithelium)

Grade of defect	Cell shedding events	Local barrier dysfunction
I (Normal)	Cell shedding confined to single cells per shedding site	None
II (Functional defect)	Cell shedding confined to single cells per shedding site	Luminal fluorescein leak or fluorescein plumes
III (Structural defect)	Microerosions in any field (lamina propria is exposed to the lumen with multiple cells being shed per site)	Luminal fluorescein leak or fluorescein plumes

Table 3.1. Watson score for CLE assessment of local intestinal barrier dysfunction. Taken from Kiesslich et al., 2012.

In patients in clinical remission, a Watson score of II or III is associated with clinical relapse at one year (Kiesslich et al., 2012), and an increased density of epithelial cell defects (microerosions) is associated with an increased risk of hospitalisation or surgery (Turcotte et al., 2012). One of the strengths of the Watson score is its excellent inter-observer reproducibility (Cohen κ 0.87).

Crohn's disease endomicroscopic activity score (CDEAS) (Neumann et al., 2012)

Using CLE in 54 patients with known colonic Crohn's disease with and without endoscopically active disease as assessed by the Crohn's disease endoscopic index of severity (Mary and Modigliani, 1989), the authors created a semi-quantitative score which correlated with CRP (taken as a marker of Crohn disease activity). Prospective validation has not been undertaken. Of the six endomicroscopic features studied, microerosions, hypervascularity and lamina propria cellular infiltrates are potentially applicable to small intestinal pathology. Interobserver agreement for these abnormalities was κ 0.51 for microerosions, κ 0.75 for vascularity (intramucosal fluorescein leak) and κ 0.83 for cellular infiltrate. On the other hand, colonic crypt tortuosity, crypt number and goblet cell number are likely to be significantly different in small bowel compared to colonic disease.

Terminal ileal microerosion density in IBD (Liu et al., 2011)

The authors manually counted epithelial 'gaps' in endoscopically normal terminal ileum in both Crohn's and UC patients, equivalent to pathological microerosions rather than single cell shedding events. Gaps per thousand enterocytes in side-on villi were manually counted to create a measure of epithelial gap density. Gap density was significantly elevated in patients with IBD compared to normal controls, although this was not related to the presence / absence of clinical disease. Paradoxically, a lower gap density was associated with a higher risk of hospitalisation in the preceding year, possibly reflecting deeper remission following more aggressive treatment in this group of patients. Prospective validation is awaited. Interobserver agreement was not assessed. Gap (microerosion) density is not a satisfactory measure of intestinal barrier dysfunction due to the lack of prospective validity, lack of discriminatory ability between inactive and active disease (when there is known to be impaired intestinal barrier integrity), unknown interobserver agreement, as well as being extremely time consuming to assess.

3.3.3.2 Irritable bowel syndrome (IBS) (Fritscher-Ravens et al., 2014)

These authors observed plumes, fluorescein leak, microerosions, intraepithelial lymphocytosis (>25/100 enterocytes) and widening of the intervillous space in response to direct small intestinal administration of food antigens in a subset of patients with suspected food intolerance / IBS. Excluding the food antigens which provoked a positive endomicroscopic result resulted in improved symptom scores, compared to those patients and controls in whom no changes were observed.

3.3.3.3 Confocal coeliac score (Leong et al., 2008)

The authors describe an excellent correlation between endomicroscopic and histological villous atrophy and crypt hypertrophy in patients with suspected / confirmed coeliac disease. Interobserver agreement in the endomicroscopic diagnosis of villous atrophy and crypt hypertrophy was good (κ 0.67 and 1.0 respectively). Other markers of enteropathy were not assessed. Although CLE was

confidently able to diagnose or exclude coeliac disease, it is not designed to assess severity of disease and is thus unsuitable as an assessment tool in EE.

3.3.4 CLE for the assessment of EE: proof of concept

In summary, discriminatory features of the CLE scoring systems described above have included

- Vascularity
- Intramucosal leak
- Luminal leak
- Distortion
- Epithelial defects (particularly microerosions).

Of these, only epithelial defects and luminal leak have been shown to be reproducible and prognostic. Intramucosal leak is reproducible and may be a marker of severity of mucosal inflammation.

Similarly, in previous work in this population we demonstrated that there was strong interobserver agreement for fluorescein leakage ($\rho = 0.94$; $P<0.0001$) and plumes ($\rho = 0.83$; $P<0.0001$), but weaker for epithelial defects such as single cell defects ($\rho =0.41$; $P = 0.01$) or micro-erosions ($\rho =0.46$; $P=0.004$) (Kelly et al., 2016). In this study we also observed that the incidence of pathological fluorescein leak in adults with environmental enteropathy was extremely high, with most fields demonstrating large amounts of intraluminal or intramucosal fluorescein. Furthermore, we have demonstrated that pathological leak observed with CLE is associated with pathological TJ protein expression (specifically claudin-4) (Kelly et al., 2016).

In this previous study, construction of a '*Z*-stack' by moving the plane of focus and recording images parallel to the mucosa (Kiesslich et al., 2012) demonstrated high levels of fluorescein leak at all observed levels, and therefore did not demonstrate enhanced discriminatory ability to detect abnormal endomicroscopic pathology. In other words, the endomicroscopic defects were so florid and universal that it was possible to assess for the presence of pathological leak in a single plane.

A modified Watson score (including intramucosal fluorescein leakage) was therefore used for the purposes of this study. The most superficial plane was selected for assessment.

3.3.5 CLE examination technique

Participants arrived in the endoscopy unit following an overnight fast and were consented for the procedure. Participants were cannulated and positioned semi-prone in the left lateral position with pulse oximetry monitoring. Participants underwent CLE under conscious sedation using pethidine (25-50mg) plus midazolam (2.5-5mg) or diazepam (2.5-10mg) intravenously.

A baseline white light endoscopy was performed. Endoscopic technique is identical to standard upper GI endoscopy (Haycock et al., 2014), although the confocal endomicroscope has reduced manoeuvrability.

The stomach was aspirated to dryness. Up to 2ml of gastric secretions for pH measurement was aspirated through the biopsy channel, which was then flushed with 100mL of air. The endoscope was advanced as far as possible into the duodenum, and once in a stable position, duodenal secretions were aspirated, taking care not to damage the duodenal mucosa by aspirating. This was followed by instillation of the carbohydrate test solution (for urinary sugar recovery assay of intestinal permeability), which was flushed through with 50mL of water. This also provided a uniform intraluminal osmotic load, as luminal leakage (and therefore observable fluorescein fluorescence) is influenced by the osmolarity of luminal contents (Kiesslich et al., 2012).

Laser settings were fixed for all procedures (gamma 1.74; gain 0.5; laser power 300-400mW). 5-10mL of 2% fluorescein was then administered intravenously. The tip of the endoscope was manoeuvred in a helical manner around the duodenal wall, from distal to proximal, for a period of 5 minutes, recording 300-400 confocal images, in order to obtain a luminal survey of the duodenal mucosa. Great care was taken to avoid areas of scope trauma and any macroscopically evident defects (e.g. duodenitis; erosions; ulcers).

Recorded images were reviewed by an experienced single investigator (JLA) for image adequacy. Duplicate or near-duplicate images, images with significant image artefact; and images <50% mucosa were discarded (Figure 3.5).

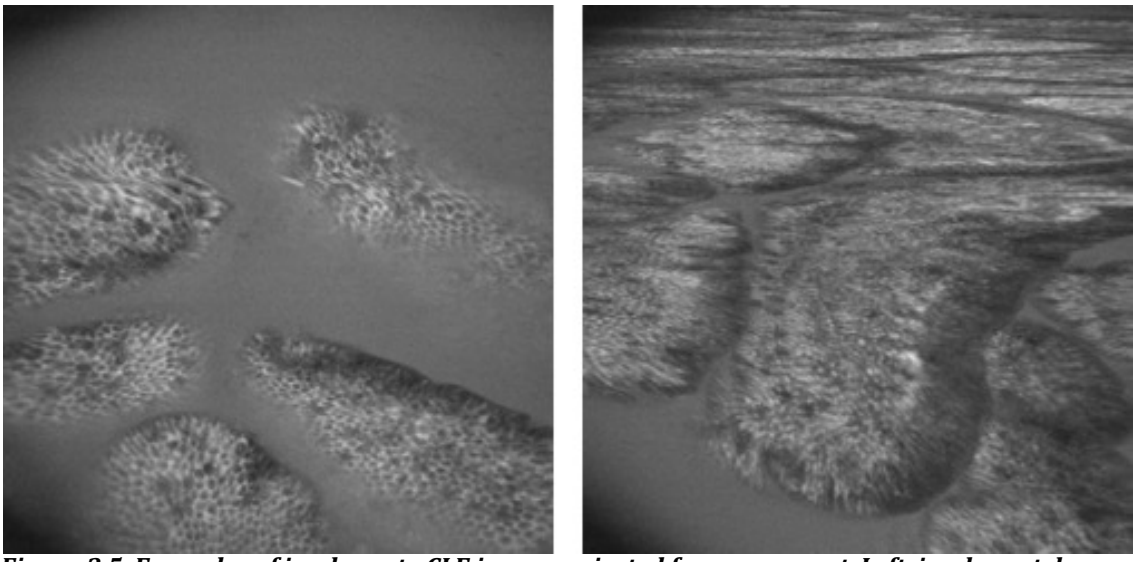

Figure 3.5. Examples of inadequate CLE images rejected for assessment. Left, inadequately visualised mucosa. Right, excessive movement artefact.

The remaining images, representing unique fields, were independently scored by two investigators (PK and JLA). The proportion of fields demonstrating local barrier dysfunction (Watson score II or III) or intramucosal fluorescein leak was recorded (Figure 3.6 and Table 3.1).

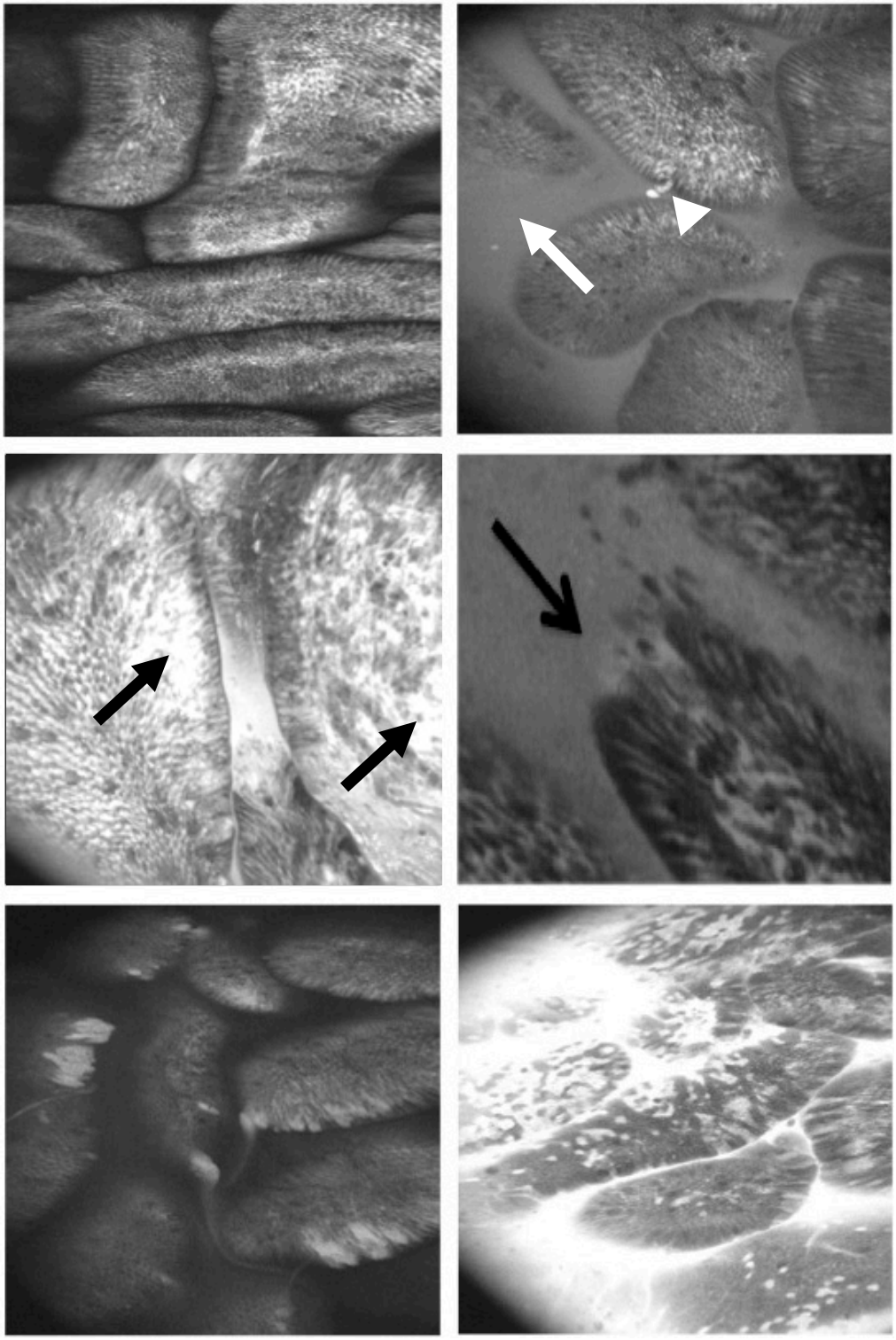

Figure 3.6. Use of CLE to assess intestinal barrier dysfunction. Examples of endomicroscopic findings with high inter-observer agreement are shown. Top left: intact barrier; no epithelial defects; no fluorescein plumes; intervillus space is black (Watson grade I). Top right: single apoptotic cell and defect (arrowhead) with increased background fluorescein leak (arrow) (Watson grade II). Middle left: marked intraluminal fluorescein leak (Watson grade III); intramucosal fluorescein leakage is also evident (arrows). Middle right: microerosion (arrow) with background fluorescein leak (Watson grade III). Bottom left: multiple plumes and microerosions without background fluorescein leakage (Watson grade II). Bottom right: florid luminal fluorescein with multiple large microerosions (Watson grade III).

CLE could provide a three dimensional image of in real-time of abnormal enteropathic small bowel mucosa. CLE identified some of the grossly distorted architecture observed histologically (Figure 3.7).

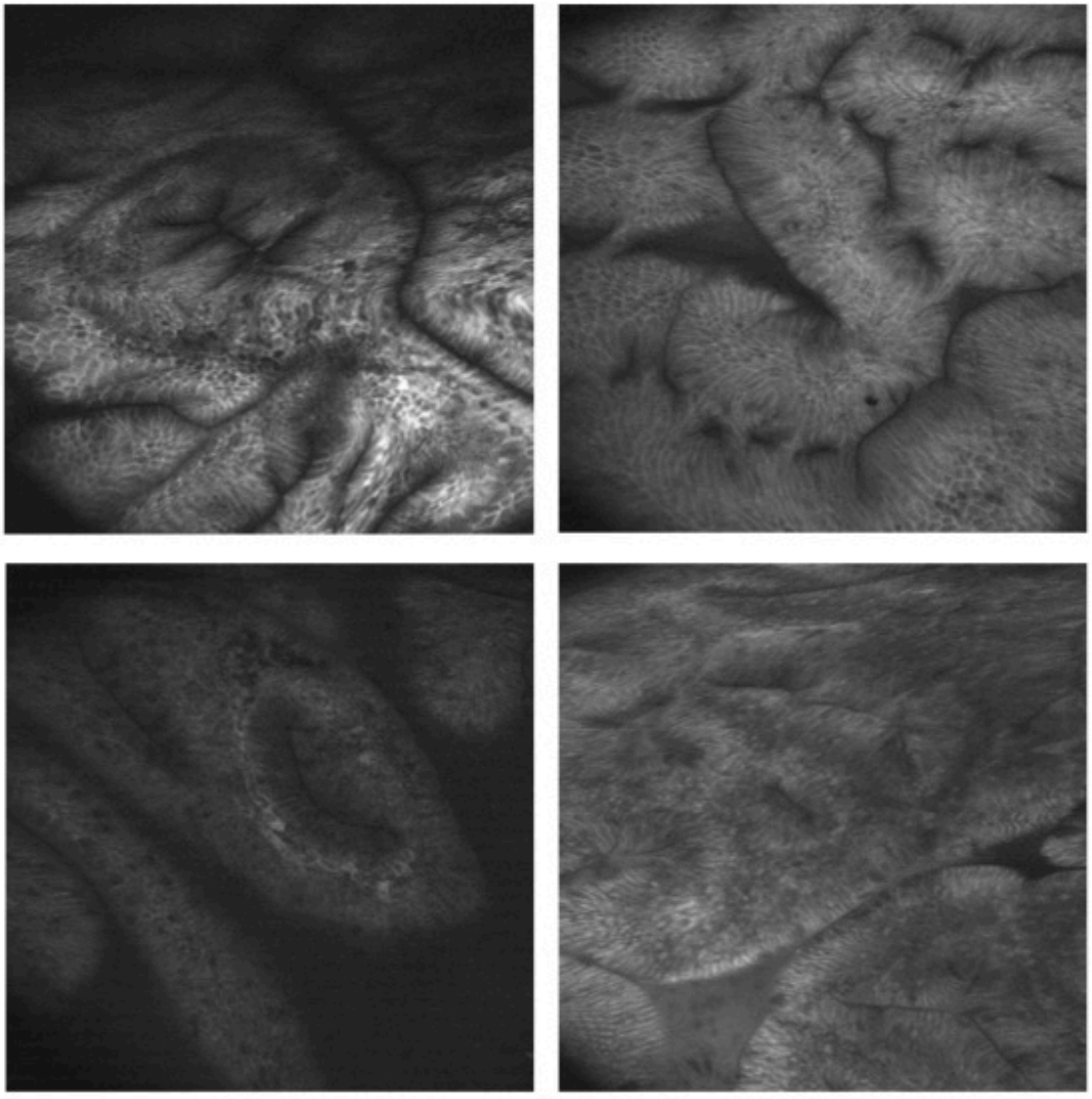

Figure 3.7. Examples of bizarre enteropathic mucosa identified by CLE in vivo. Mucosal bridges and villus fusion are clearly demonstrated.

3.4 Assessing lamina propria T cell MTORC1 activity by flow cytometry

3.4.1 Principle

Flow cytometry allows the identification and quantification of antigens of interest simultaneously at the resolution of single cells. Most commonly this is used to immunophenotype a population of cells, but other common uses include monitoring cell signalling pathways in response to stimulation or inhibition.

For cellular analyses, a solution of cells is prepared with antibodies directed against antigens of interest. Each antibody is conjugated directly or indirectly to a fluorochrome with an emission spectrum which differs from every other fluorochrome being used. Cells then pass through a laser array in single file. Bound antibody-fluorochromes on each cell are excited by a laser at an appropriate excitation wavelength and emit light at their known spectra. Sensors detect this emitted light, with each emission spectrum corresponding uniquely to one antigen of interest, so that the intensity of emitted light which is detected is proportional to the amount of antigen present in each cell. In practice, however, fluorochrome emission spectra are relatively broad, and this usually results in 'spectral overlap' – that is, light emitted by one fluorochrome coincides with light emitted from another over part of its emission spectrum.

If unaccounted for, this overlapping light emission will be misinterpreted as a positive signal from a second fluorochrome, when in fact it merely represents a positive signal from the first. This is corrected by compensation. To compensate for FITC in the PE channel in the example above, a sample stained with FITC alone is analysed. The proportion of emitted light in the grey overlap would be detected in the PE channel (where there is no true PE signal). This 'false positive' signal can be mathematically neutralised, so that after compensation the signal in the PE channel from FITC is minimised (Figure 3.8). This compensation can then be applied to samples where FITC and PE (in this case) are used together.

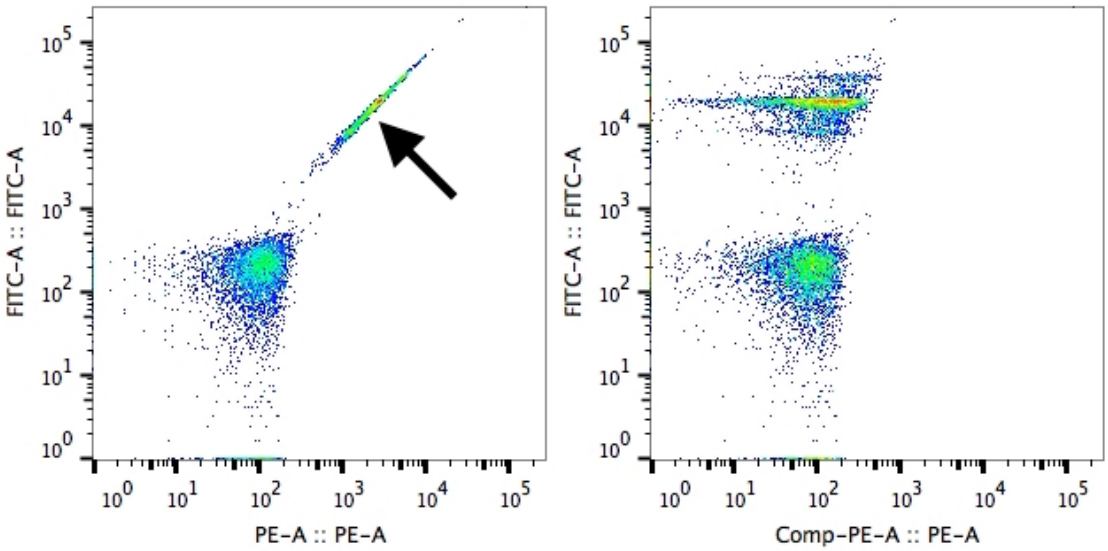

Figure 3.8. Principle of compensation. In the left panel, FITC-conjugated compensation beads (arrow) are incorrectly detected in the PE channel (x axis) due to spectral overlap, resulting in a false positive PE signal. After compensation (right panel), these FITC-conjugated beads no longer appear positive in the PE channel (mathematically, the median fluorescence intensity values of FITC positive and negative populations in the PE channel have been equalised).

3.4.2 Specific considerations in the flow cytometric analysis of MTORC1 activity

The assessment of MTORC1 activity by flow cytometry in mucosal lymphocytes presents a number of technical challenges in addition to those required for conventional flow cytometry. Directly conjugated antibodies to phospho-4EBP1 (T36/T45) – the initial sites of phosphorylation by activated MTORC1 (Gingras et al., 1999) – are commercially available, and have been used in a small number of published experiments using human or murine PBMCs or cultured cell lines (Hoerning et al., 2015; Syed Alwi et al., 2010; Zeng et al., 2013, 2012). However, the assessment of MTORC1 activity in LPMCs by flow cytometry has not been reported.

3.4.2.1 Intracellular antigen localisation

MTORC1 and its targets are intracellular proteins, and thus require cell membrane permeabilisation to become accessible to antibodies. All published and

manufacturer's protocols use methanol (or proprietary methanol-containing buffers) as the permeabilising agent, at concentrations varying from 50% to 90%. Methanol also has a fixative effect on cellular proteins; methanol-permeabilised cells can be stored at –20°C for several months.

However, methanol is a harsh alcohol and has significant denaturing effects. This is particularly relevant in flow cytometry where exposure of antigens to methanol can destroy the reactive epitope, thus reducing the staining intensity, and therefore brightness, of a given antigen. This effect is epitope-dependent, and therefore some clones are known to be methanol sensitive or insensitive (for example, CD3 clone UCH-T1 is relatively methanol resistant, whereas clone HIT-3A is methanol sensitive). The methanol sensitivity of many antibody clones is unknown. On the other hand, intracellular antigens in particular may benefit from more aggressive permeabilisation in order to increase the amount of antigen available for antibody binding.

In preliminary work using Ficoll-isolated human PBMCs, permeabilisation using methanol greater than 50% resulted in marked loss of CD3 (clone UCH-T1) signal, without improving phospho-4EBP1 signal (Figure 3.9). 50% methanol was therefore selected as the permeabilising agent for the study.

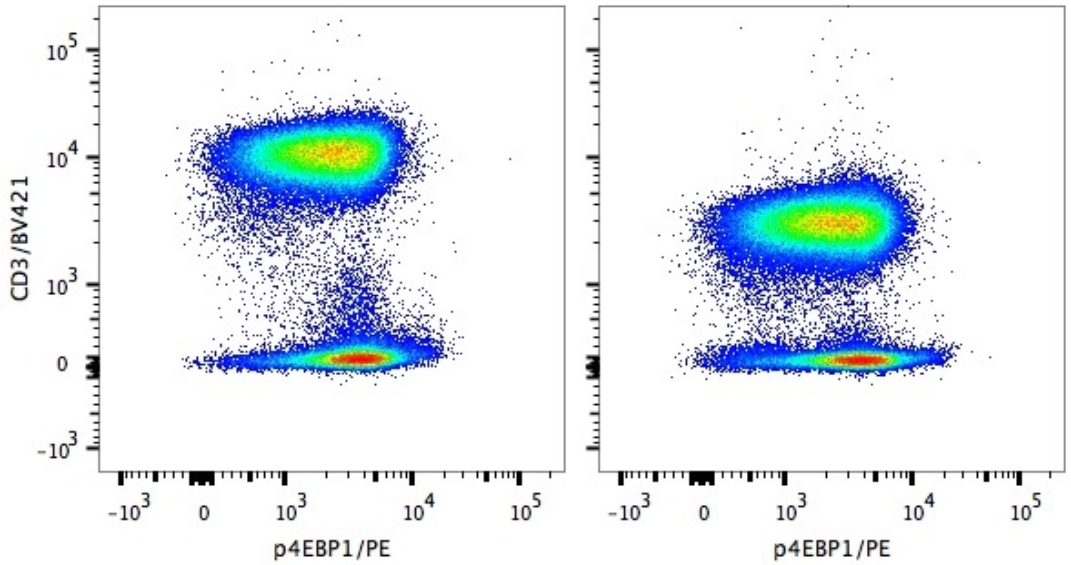

Figure 3.9. Effect of methanol concentration on CD3 and phospho-4EBP1 staining. Staining was performed after permeabilisation with 50% (left panel) or 70% (right panel) methanol.

3.4.2.2 Phosphorylation and dephosphorylation kinetics

MTORC1 activity is reflected in levels of phospho-4EBP1. Phosphorylation events in response to a stimulus are in general transient and relatively short-lived, and can therefore be missed. In preliminary work using Ficoll-isolated human PBMCs from a healthy adult UK resident, the phosphorylation kinetics of 4EBP1 in freshly isolated $CD3^+$ lymphocytes were studied (Figure 3.10). Phosphorylation levels did not increase from baseline levels despite stimulation with a variety of nutrient rich media, reflecting attenuation and accommodation of the MTORC1 pathway in response to a nutrient stimulus.

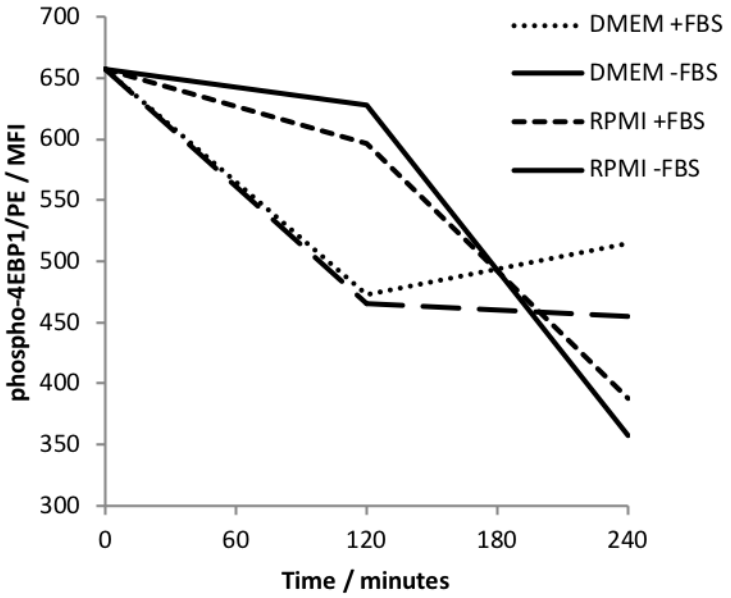

Figure 3.10. 4EBP1 phosphorylation kinetics in freshly isolated $CD3^+$PBMCs stimulated with DMEM or RPMI media, with or without foetal bovine serum (FBS).

In a second experiment, freshly Ficoll-isolated PBMCs from four healthy adult UK residents were cultured in a nutrient deplete medium (HBSS with 4mM glucose). At two hours, phospho-4EBP1 levels were approximately 50% of baseline, and little further dephosphorylation occurred up to four hours (Figure 3.11).

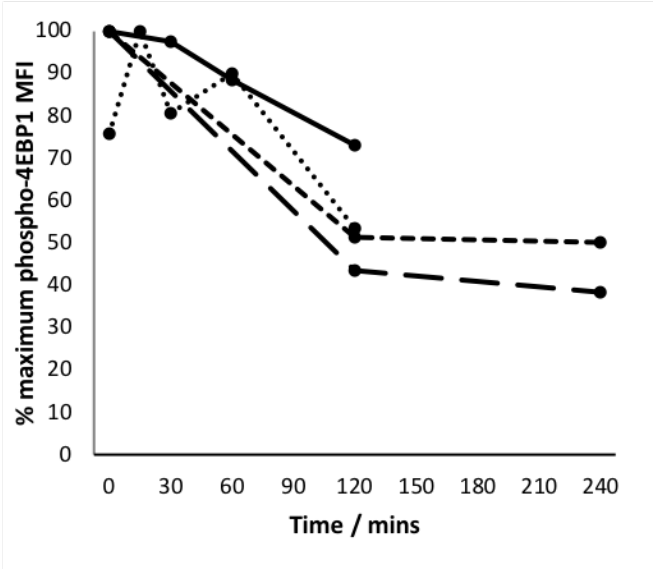

Figure 3.11. Phospho-4EBP1 dephosphorylation kinetics in freshly isolated CD3⁺PBMCs cultured in HBSS. Data from four healthy adult UK residents.

Finally, the 4EBP1 phosphorylation kinetics of $CD3^+$ PBMCs from basal levels were assessed by stimulating them with nutrient rich RPMI complete medium following a two hour period of nutrient deprivation in HBSS with 4mM glucose. This demonstrated that maximal phosphorylation occurs between one and two hours, with near-maximal phosphorylation levels sustained for at least four hours (Figure 3.12). This is in keeping with other published *in vitro* data (Burnett et al., 1998; Von Manteuffel et al., 1996).

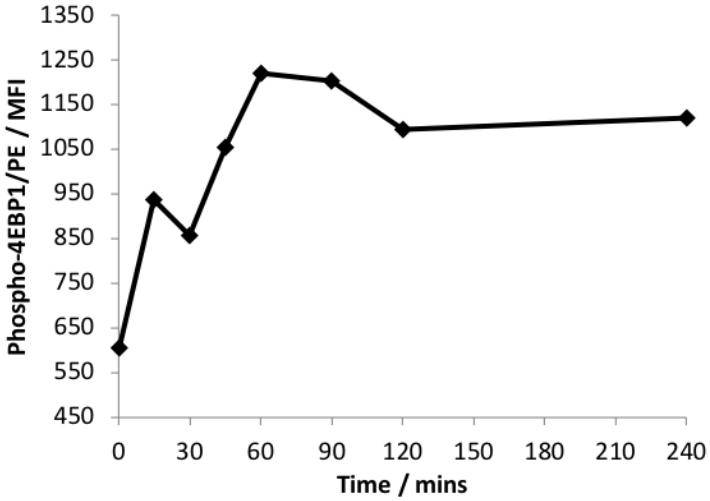

Figure 3.12. 4EBP1 phosphorylation kinetics in PBMCs in response to nutrient stimulation following two hour nutrient deprivation.

On the basis of this, lamina propria lymphocytes were deprived of nutrients for 90 minutes after isolation before being stimulated in nutrient-rich complete medium for 90 minutes.

3.4.2.3 Low levels of phosphoprotein expression

Under normal conditions, cellular kinases are constitutively inactive, with kinase activation furthermore being generally short lived. Protein dephosphorylation also occurs concurrently. These processes usually result in low levels of phosphorylated as compared to the dephosphorylated proteins. A common notable exception is in cancer, where mutations commonly result in constitutively active kinases and relatively high levels of target phosphoproteins.

Since the magnitude of antibody-fluorochrome signal is directly related to the level of target antigen present (in this case phosphoprotein), phosphoprotein signals in flow cytometry tend to be 'dim'. This results in two important technical considerations:

1. The antibody should be conjugated to the brightest fluorophore possible (a phospho-4EBP1 – PE conjugate was therefore chosen).
2. Care should be taken to ensure that spectral overlap from other colours in the panel do not diminish the target signal (FITC was therefore avoided).

3.4.2.4 Determination of positivity

Many antigens (such as CD3 and CD4 on lymphocytes) have a bimodal expression pattern, with very few cells expressing intermediate levels. This results in clearly defined positive and negative populations. However, many antigens are expressed at variable levels, meaning that it is not immediately possible to divide a population into positive and negative. In addition, phenomena such as autofluorescence and non-specific antibody binding mean that cells that do not express an antigen of interest may nonetheless exhibit detectable fluorescence which may be incorrectly be interpreted as being positive for the target antigen. Determination of what constitutes a 'positive' for an antigen with a non-bimodal

distribution therefore generally requires the use of one or more of the following controls (Maecker and Trotter, 2006).

Biological controls.

Particularly when assessing response to stimulation, the most relevant control is usually an unstimulated/baseline/negative control sample, which will simultaneously control for non-specific antibody binding and fluorescence spillover. Any fluorescence above that observed in the negative sample must be attributable to the effects of stimulation. A biological (unstimulated) paired control for the p4eBP1 readout was therefore used. The percentage of cells responding to a stimulus (in this case p4eBP1 positive cells) can be calculated by subtracting the unstimulated / control histogram (in this case, nutrient starved cells) from the stimulated histogram (in this case, nutrient stimulated cells). A number of mathematical algorithms exist, of which the proprietary Super-enhanced DMax (SED) method is thought to be most accurate (Bagwell, 1996). The biological controls for phospho-4EBP1 expression in the following experiments were the nutrient-deprived lymphocytes.

Isotype controls.

Isotype controls are non-specific antibodies of the same isotype class as the test antibody. Use of isotypes theoretically allows determination of non-specific background staining, which must be subtracted from the staining observed with the specific (target) antibody, to give the amount of staining attributable to specific antibody binding. Most isotype controls have only been tested and validated with extracellular antigens; intracellular staining (following permeabilisation) may be significantly greater, and in fact may not be non-specific. Furthermore, to be truly comparable, the antibody-fluorochrome characteristics of the isotype (e.g. fluorochrome : antibody ratio) must match the target antibody; perfectly matched isotypes are rarely possible. In the following experiments, isotype controls for HLA-DR and TBET staining were used as suggested and validated by the manufacturer.

Fluorescence-minus-one (FMO) controls.

In multi-colour experiments with variable target antigen staining intensity, fluorescence spillover effects may be complex. This can be controlled by the use of FMOs. FMOs allow the measurement of total background fluorescence in a given channel when all antibodies/fluorochromes are included with a sample except the antibody/ fluorochrome in that channel (i.e. all antibodies minus one). Any measured fluorescence in this channel can then be attributed to non-specific background fluorescence (including autofluorescence). When the antibody of interest in added (i.e. the full panel), any fluorescence above this value must be due solely to the antibody of interest. FMOs cannot provide any information on non-specific antibody staining (which is ususally provided by isotype controls); however it has been argued that with modern, high quality antibodies, the majority of background / non-specific staining is due to spillover effects, rather than non-specific antibody binding.

3.4.2.5 Effect of LPMC isolation on phosphostatus

The time and unphysiological processes involved in isolating LPMCs from biopsy tissue (whether enzymatically or by biopsy culture) mean that the isolated cells will not reflect *in vivo* phospho-4EBP1 status. It is therefore more appropriate to measure the phosphorylation response to a stimulus, on the reasonable assumption that the isolated cells (particularly if isolation occurs soon after biopsy) will retain much of the phenotype and therefore the function of their *in vivo* counterparts.

3.4.2.6 Limited tissue and lamina propria lymphocyte availability

LPMCs were isolated from up to four endoscopic duodenal biopsies per participant. In preparatory work, mean yield following fixation and methanol permeabilisation was up to approximately 10000 lymphocytes per biopsy at most. Less aggressive permeabilisation (for example for TBET staining) improved lymphocyte yield by a factor of 5-10. This relatively small number of lymphocytes

meant that examining phospho-4EBP1 expression had to be limited to common cell populations.

3.4.2.7 Effect of methanol on fluorochrome-conjugated antibodies

Many fluorophores are protein-based, and are therefore susceptible to degradation by methanol in the permeabilisation step. Many fluorophores can therefore not be used before a methanol permeabilisation step (i.e. in a stain-fix-perm protocol). Furthermore, the amide linkages in tandem dyes are also susceptible to degradation. This would result in a false positive signal in the 'donor' channel (e.g. from APC-Cy7 degrading to APC). A 1-step fix-perm-stain protocol was utilised, as many of the fluorochromes used were methanol sensitive.

3.4.2.8 Choice of stimulus for MTORC1 pathway

Nutrients, and amino acids in particular, are the canonical agonists of the MTORC1 pathway. Common cell culture media contain relatively high levels of essential and non-essential amino acids, and thus provide an easily reproducible stimulus. Common media such as RPMI and DMEM contain 2-fold differences in amino acid concentrations, but preliminary work suggested that the higher concentrations in DMEM did not significantly enhance MTORC1 activity compared to RPMI.

3.4.2.9 Resource limitations

Access to technical support in Zambia is limited, with online support and servicing being provided from South Africa, approximately 1200km away. Furthermore antibodies and reagents had to be imported from the UK by freight. Panels and experiments were therefore designed to be technically robust. For example, once fixed and permeabilised, cell samples could be stored in 50% methanol at –20°C for several weeks if required before staining and analysis without significant antigen degradation. The 2-laser FACSVerse cytometer (BD Biosciences, UK)

available in Zambia supported a maximum of six colours which also significantly influenced panel design.

3.4.3 Assay principle

The responsiveness of lamina propria lymphocytes to nutrient stimulation can be assessed *in vitro* by measuring the percentage of cells that produce phospho-4EBP1 in response to nutrient stimulation compared to nutrient deprivation. These results can determine (1) whether the mucosal immune compartment response to nutrient stimulation differs according to severity of EE; and (2) whether nutrient supplementation alters the responsiveness of the MTORC1 pathway to nutrient stimulation. Furthermore, basal and maximal levels of MTORC1 activity can be measured simultaneously.

3.4.4 Final antibody panel

Taking these considerations into account, the following antibody panel was designed (Table 3.2). The predicted emission spectra are shown in Figure 3.13, demonstrating that the phospho-4EBP1 antigen conjugated to the 'bright' PE fluorophore is minimally affected by spillover from the other fluorophores in the panel.

	Immunophenotype	**Conjugate**	**Determination of positivity**
Activated MTORC1	p4EBP1$^{(T36/T45)+}$	PE	Unstimulated control
Activated cell	HLA-DR^{+}	APC	IgG_{2A} κ isotype control
T lymphocyte	CD3^{+}	APC-Cy7	Bimodal
CD4^{+} lymphocyte	CD3^{+} CD4^{+}	PerCP-Cy5.5	Bimodal
CD8^{+} lymphocyte	CD3^{+} CD4^{-}	–	–

Table 3.2. Antibody panel for the MTORC1 activity assay.

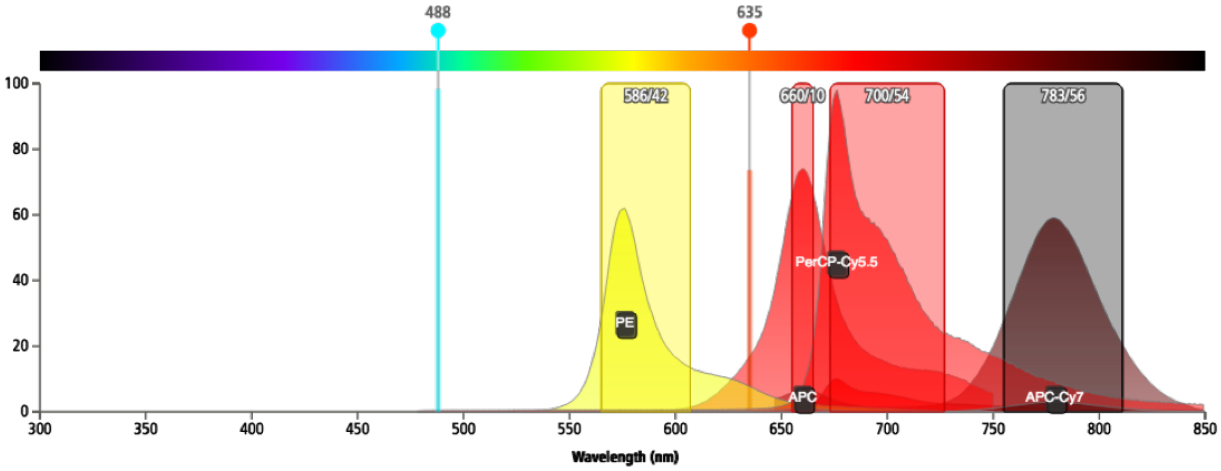

Figure 3.13. Predicted emission spectra for the panel, with lasers at 488nm & 635nm. Created using BD Biosciences spectra viewer (www.bdbiosciences.com/us/s/spectrumviewer)

3.4.5 Assessment of lamina propria CD3⁺ lymphocyte MTORC1 activity in response to *in vitro* nutrient deprivation and stimulation: method

Lymphocyte isolation

3-4 endoscopic distal duodenal biopsies were taken at the end of the endoscopy procedure and placed in 1ml of RPMI complete medium on ice for up to 4 hours, until ready for processing. Mucus and debris were removed by gentle agitation with 5ml plain HBSS in a Petri dish. The biopsies were then placed in 10ml pre-warmed 1mM EDTA, and agitated vigorously on a heater/shaker at 37°C for 15 minutes to remove the epithelium.

Lamina propria mononuclear cells (LPMC) were then enzymatically liberated from the remaining lamina propria using an enzymatic digestion solution made up immediately before use consisting of 1mg/mL collagenase D (Roche Diagnostics GmBH, Mannheim, Germany) and 20µg/mL DNAse I (Roche Diagnostics GmBH, Mannheim, Germany) in pre-warmed complete RPMI. The remaining lamina propria were transferred to a Petri dish with 250µl of enzymatic digestion solution, to prevent desiccation. The tissue was rapidly macerated with scalpels to create a finely particulate solution. Tissue retrieval was maximised by rinsing the scalpel blades with enzymatic digestion solution, and by rinsing the Petri dish. The

macerated tissue was placed into a total of 10ml of pre-warmed enzymatic digestion solution. The lamina propria / digestion solution was vigorously agitated for 40 minutes on a heater/shaker at 37°C.

The resulting suspension containing LPMCs was filtered through a 100μm cell strainer, with the digestion vessel rinsed with plain HBSS, to give a total volume of 20ml. The cell suspension was centrifuged at 400*g* for 5 minutes and the supernatant discarded. The cell pellet was resuspended in 4mL complete pre-warmed RPMI and divided into 2 aliquots. Both aliquots were centrifuged once more at 400*g* for 5 minutes and the supernatant discarded.

MTORC1 inhibition and stimulation

Both aliquots were resuspended in 2ml pre-warmed HBSS supplemented with 1mM L-Glutamine and incubated for 90 minutes at 37°C. One aliquot (representing the nutrient deprived / negative control / basal phospho-4EBP1 condition) was then fixed with an equal volume of 8% PFA (to give a total concentration 4%) for 10 minutes on ice. Fixed cells were centrifuged at 400*g* for 5 minutes and the supernatant discarded. The cell pellet was resuspended in 3mL FACS buffer (Biolegend UK), centrifuged once more at 400*g* for 5 minutes and the supernatant discarded. The pellet was resuspended in the residual FACS buffer by vortexing then placed on ice for 5 minutes. Cells were fixed with 50% MeOH at –20°C and stored at least overnight at –20°C until staining and analysis.

The second aliquot (representing the nutrient stimulated / maximal phospho-4EBP1 condition) was resuspended in 2ml pre-warmed complete RPMI supplemented with 4mM L-Glutamine, and incubated for a further 90minutes at 37°C before being fixed in 4% PFA and permeabilised in ice cold 50% MeOH as above.

The composition of the nutrient deprivation / nutrient stimulating solutions is given in Appendix 5, p.221.

Staining, acquisition, and analysis

Cells were washed by adding FACS buffer, and then washed two further times in FACS buffer and resuspended in the residual at each step. Antibodies were added to the residual according to Table 3.3 below and incubated at room temperature in the dark for one hour. The staining mixture was washed with FACS buffer once, then the cells were acquired immediately in 300µl FACS buffer on a FACSVerse flow cytometer (BD Biosciences UK).

HLA-DR positivity was determined using the relevant isotype control. Automated compensation was performed using FACSuite (BD Biosciences). Gating and analysis was performed using FlowJo v.10.0.7 (FlowJo LLC, Ashland, Oregon). An example of the gating strategy used is given in Figure 3.14. The proportion of phospho-4EBP1 positive cells was calculated using the super-enhanced Dmax method (Bagwell, 1996).

	Stimulated	**Nutrient deprived / control**	**Clone; manufacturer; cat. #**
Phospho-4EBP1$^{(T36/T45)}$ / PE	1µl	1µl	Cell Signaling Technology; 2855
CD3 / APC-Cy7	2µl	2µl	SK7; BD Biosciences; 557832
CD4 / PerCP-Cy5.5	1µl	1µl	RPA-T4; BD Biosciences; 560650
HLA-DR / APC	2µl	–	G46-6; BD Biosciences; 559866
IgG_{2A} κ isotype / APC	–	2µl	G155-178; BD Biosciences; 555576

Table 3.3. Antibodies used for MTORC1 assay.

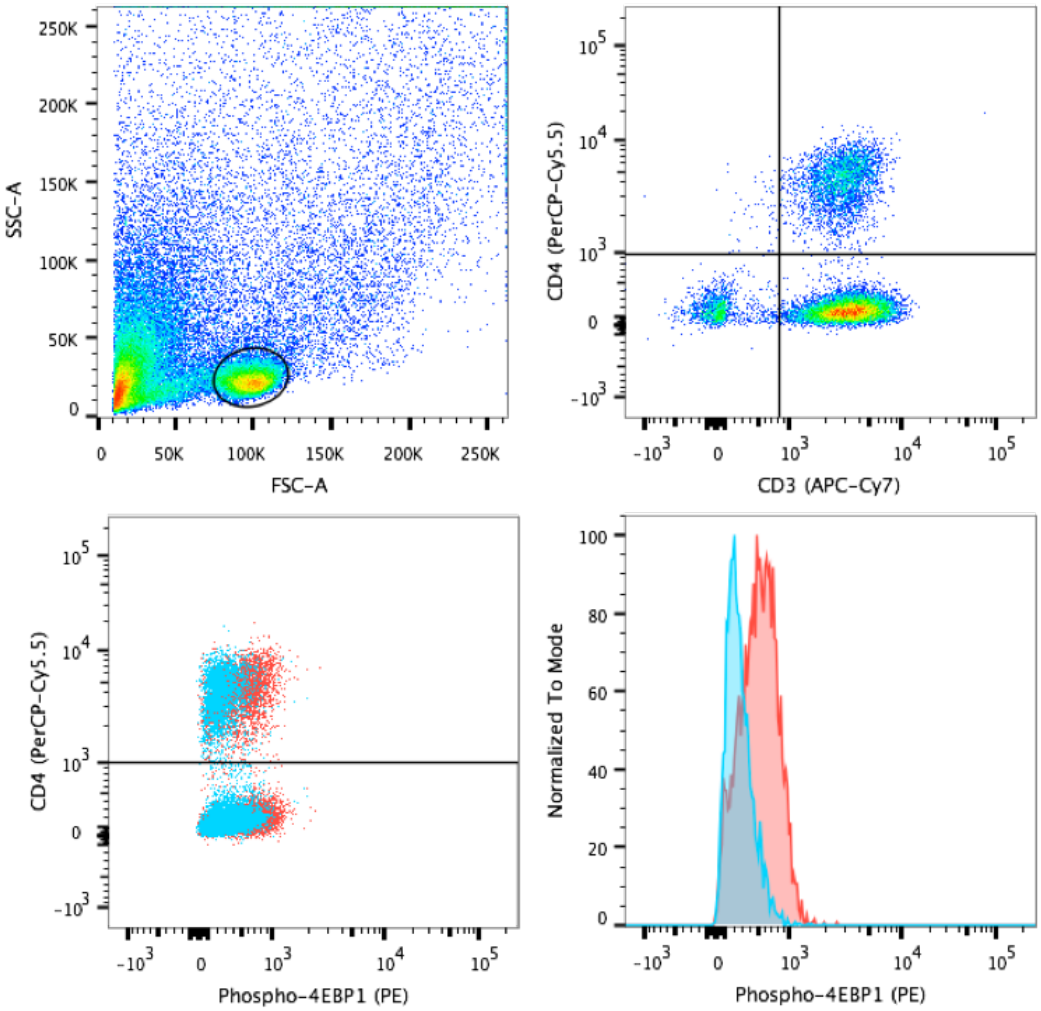

Figure 3.14. Gating strategy for determining phospho-4EBP1 positivity. Top left, lymphocyte gate. Top right. CD3⁺ CD4⁺ gate. Bottom left, p4EBP1 in unstimulated (blue) and stimlulated (red) conditions. Bottom right, p4EBP1 in unstimulated (control, blue) and stimulated (red).

3.5 Identification and quantification of lamina propria T_{H1} cells

3.5.1 Principle

The *TBET* ('T-Box Expressed in T cells') gene encodes the TBET protein which is required for the expression of the canonical T_{H1} cytokine IFNγ. Classical T_{H1} cells are phenotypically $CD3^+$ $CD4^+$ $TBET^+$, and can be identified using antibodies directed against these antigens. The proportion of T_{H1} LP cells was assessed after the supplementation period.

3.5.2 Method

The TBET stain was incompatible with methanol permeabilisation, which was required for adequate p4eBP1 staining (Figure 3.15). It was therefore not possible to assess p4EBP1 and TBET status in the same panel. A separate aliquot of isolated LPMCs was therefore used for assessing TBET status.

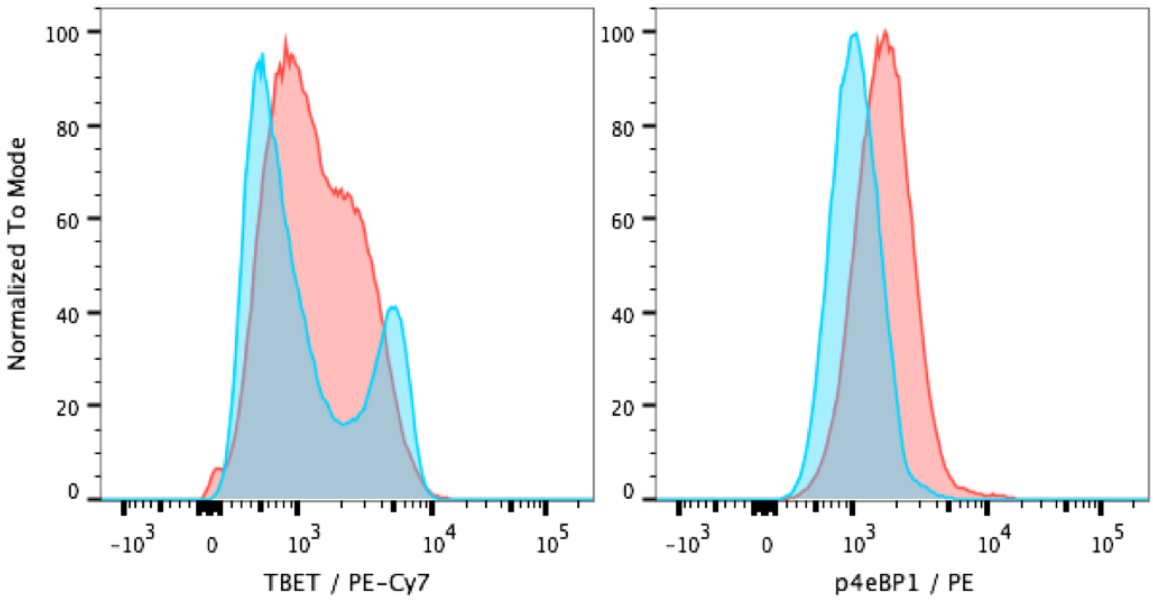

Figure 3.15. Incompatibility of TBET (blue histograms) and phospho-4EBP1 (red histograms) fixation / permeabilisation protocols. Illustrative experiment from human PBMC sample. Left panel, TBET staining. Right panel, phospho-4EBP1 staining.

In practice and in contrast to PBMCs, LP lymphocytes did not demonstrate a bimodal distribution pattern so an isotype control was used to determine positivity (Figure 3.16).

LPMC were mechanically and enzymatically isolated as for MTORC1 analysis (section 3.4.5, p. 122). After filtration, washing and resuspension, samples for TBET analysis were divided into two further aliquots, centrifuged at 400*g* for 5 minutes at room temperature and the supernatant discarded. The pellet was resuspended in 0.5ml Fix/Perm solution (eBioscience UK), vortexed thoroughly, and incubated at room temperature for 45 minutes. Without washing, 1ml of Perm Buffer (eBioscience UK) was added, and the mixture centrifuged at 400*g* for 5

minutes. The supernatant and residual were discarded and the pellet resuspended in 100µl Perm Buffer and 2.5µl FBS.

The antibody panel is given in Table 3.4. Antibodies were added according to Table 3.5 and incubated at room temperature in the dark for one hour. Cells were then washed twice in Perm Buffer, then resuspended in 300µl FB and acquired on a FACSVerse cytometer.

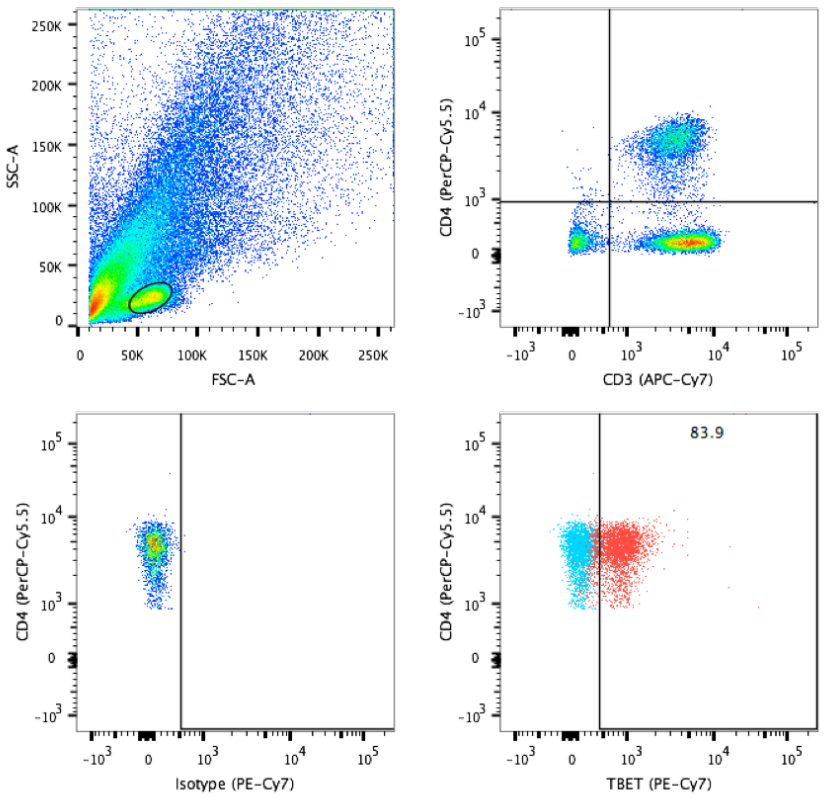

Figure 3.16. Gating strategy to identify T_{H1} ($CD3^+$ $CD4^+$ $TBET^+$) lymphocytes.

	Immunophenotype	Conjugate	Determination of positivity
T_{H1}	$CD3^+$ $CD4^+$ $TBET^+$	PE-Cy7	IgG_1 κ isotype control
Activated cell	$HLA\text{-}DR^+$	APC	IgG_{2A} κ isotype control
T lymphocyte	$CD3^+$	APC-Cy7	Bimodal
$CD4^+$ lymphocyte	$CD3^+$ $CD4^+$	PerCP-Cy5.5	Bimodal

Table 3.4. Antibody panel for T_{H1} assay.

	T_{H1} assay	Control	Clone; manufacturer; cat. #
TBET / PE-Cy7	1µl	–	4B10; Biolegend, 644824
IgG_1 κ isotype / PE-Cy7	–	1µl	MOPC-21; Biolegend; 400126
CD3 / APC-Cy7	2µl	2µl	SK7; BD Biosciences; 557832
CD4 / PerCP-Cy5.5	1µl	1µl	RPA-T4; BD Biosciences; 560650
HLA-DR / APC	2µl	–	G46-6; BD Biosciences; 559866
IgG_{2A} κ isotype / APC	–	2µl	G155-178; BD Biosciences; 555576

Table 3.5. Antibodies used for T_{H1} assay.

3.6 Cell viability and cell permeabilisation assay

To confirm lymphocyte viability immediately before fixation, a cell viability assay was performed on 26 LP lymphocyte samples; adequacy of permeabilisation was also assessed.

LPMC were mechanically and enzymatically isolated as for MTORC1 analysis (section 3.4.5, p. 122). An aliquot of cells was taken from both the nutrient deprived and nutrient stimulated tubes at the end of the incubation period and prior to fixation.

To assess viability at the end of the incubation period, cells were centrifuged at 600*g* for 5 minutes at room temperature and the supernatant discarded. Cells were resuspended in the residual by vortexing. Cells were stained with CD3 / APC-Cy7 for 30 minutes at room temperature, washed once in FACS buffer, and resuspended in 250µl of FACS buffer. Cells were then stained with 1µl 7-AAD (BD Biosciences UK, cat. 559925) for ten minutes and acquired immediately (Figure 3.17).

The remaining cells were processed and analysed for T_{H1} or MTORC1 activity as above. To assess adequacy of permeabilisation, surplus cells after acquisition were stained with 1µl 7-AAD for ten minutes at room temperature and acquired immediately (Figure 3.17).

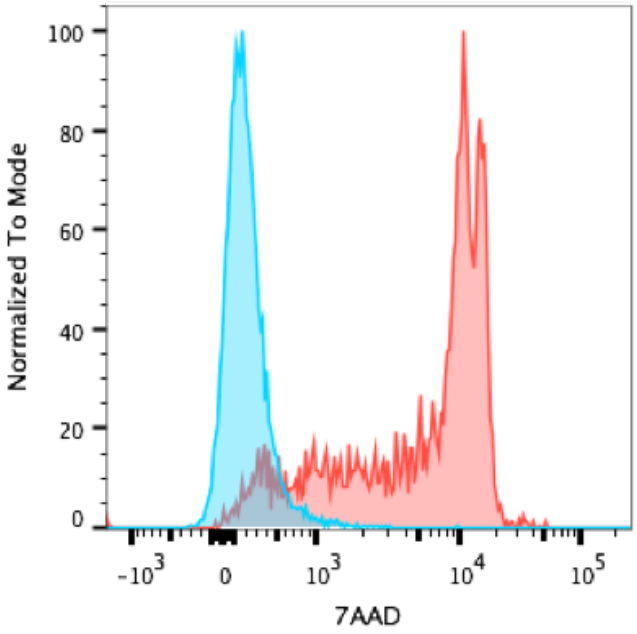

Figure 3.17. Representative experiment of 7-AAD staining in LP CD3⁺ lymphocytes before and after fixation and permeabilisation. Cells immediately before fixation were viable on the basis of 7-AAD exclusion (blue histogram). After fixation and permeabilisation, only a minority of cells did not take up 7-AAD (red histogram).

3.7 Urinary metabonomics

3.7.1 Rationale

Detailed characterisation of the microbiome does not in itself provide insights into disease mechanisms. The vast complexity and variability of the microbiota means that a global assessment of microbiota-host interactions is required. The microbiota interacts with the host primarily through metabolic and immunological pathways. Metabonomics is a powerful technique which provides an integrative, functional, global evaluation of host and microbial interactions through measuring their co-metabolites, and which can be used to identify disease, response to therapy, and functional changes in the microbiota. Metabonomics has been used to

study severe acute malnutrition, where it was shown that nutritional intervention results in changes in the metabonomic profile which reflects transferrable alterations in the microbiota in a murine model (Smith et al., 2013). There have now been some studies investigating the urinary metabonomic profile in children with EE (Farràs et al., 2018; Guerrant et al., 2016; Mayneris-Perxachs and Swann, 2018; Mayneris-Perxachs et al., 2016), but so far the adult metabonome has not been studied.

3.7.2 Principle

Metabonomics is defined as 'the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modifications' (Nicholson et al., 1999). The metabonome consists of the host's global metabolite profile, and is influenced by the host's microbiota and diet & other lifestyle factors, as well as host & microbiota metabolic pathways. These complex metabolic 'signatures' reflect processes occurring in the host and the microbiota across multiple functional compartments (Claus and Swann, 2013).

NMR spectroscopy is the most commonly used technique for detecting and analysing samples. Urine samples have the advantage of being safe and non-invasive, and reflect host-microbiota interactions through systemic absorption and excretion of gut products (Claus and Swann, 2013).

3.7.3 Method

Urinary ^{1}H NMR spectroscopic metabonomic analysis was performed by Dr Jordi Mayneris and Dr Jonathan Swann, Department of Surgery and Cancer, Imperial College London (Figure 3.18).

Urine samples were collected prior to endoscopy and immediately decanted into a container with 1 mL 0.5% chlorhexidine. Samples were stored at –80°C until transportation to London on dry ice. Urine samples (400µL) were combined with 200µL of phosphate buffer (pH 7.4; 100% D_2O) containing 1mM of 3-trimethylsilyl-1-[2,2,3,3-$^2H_4$] propionate (TSP) as an external standard and 2mM sodium azide as a bactericide. Samples were mixed by vortexing and centrifugated at 10000*g* for 10 minutes. The supernatant (550µL) was transferred to a 5 mm internal diameter NMR tube. Samples were analysed by ^1H nuclear magnetic resonance (NMR) spectroscopy using a 700MHz Bruker NMR spectrometer operating at 300K. For each urine sample a standard noesy experiment was performed with water-suppression using 8 dummy scans followed by 128 scans collected into 64K data points. A mixing time of 10ms was used with an acquisition time of 3.8s and a recycle delay of 3.0s. ^1H NMR spectra were manually corrected for phase and baseline distortions. Chemical shifts in the spectra were referenced to the TSP peak at δ 0.0ppm. Spectra were digitized using an in-house Matlab (version R2012a, The Mathworks, Inc.; Natwick, MA) script. Resonances derived from water were removed to minimize distortions to the spectral baseline. Spectra were normalized to the total area.

Figure 3.18. Metabonomics method.

3.8 Quantification of plasma CRP, sCD14 & GLP2 by ELISA

3.8.1 Introduction

A plasma biomarker panel of sCD14, CRP and LPS was selected as an adjunctive assessment of microbial translocation and immune activation. Furthermore, levels of glucagon-like peptide 2 (GLP2) were measured to assess the enterohumoral axis, in particular its response to amino acid supplementation.

The measurement of LPS is described in section 3.9, p.133.

3.8.2 Quantitative sandwich ELISA principle

Microplate wells are coated with monoclonal antibodies specific for the antigen of interest. Samples and standards are incubated in the wells, so that antigen is bound by the fixed antigen-specific antibody. The immobilised antigen-antibody complex is then incubated with a second antigen-specific antibody, which is conjugated to an enzyme such as horseradish peroxidase (HRP).

A substrate solution containing hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine (TMB) is then added. The presence of HRP catalyses a chromogenic reaction resulting in the formation of TMB diimine, which has a blue colour. The reaction is stopped with the addition of strong acid, resulting in a yellow-green colour which can be quantitatively measured by colorimetry using a spectrophotometer. The amount of TMB diimine present (the intensity of colour) is proportional to the amount of antibody-antigen-antibody conjugate present.

3.8.3 Sample preparation

Fresh whole blood was centrifuged at 1000*g* for 15 minutes at 4°C. The plasma was then aliquoted and stored at –20°C until required. ELISAs were performed according to the manufacturer's instructions, with dilutions where required as stated in Table 3.6. Samples, standards and controls were plated in duplicate. Optical densities were determined immediately after stopping with a microplate reader measuring absorbance at 450nm, with the correction wavelength set at 540nm for CRP and sCD14 and 590nm for GLP2 (EL800, BioTek UK, Swindon).

Marker	Manufacturer	Cat. #	Dilution	Standards range
CRP	R&D Systems	DCRP00	1/1000	0-50ng/ml
sCD14	R&D Systems	DC140	1/400	0-16ng/ml
GLP2	EMD Millipore Corp.	EZGLP2-37K	N/A	0-73ng/ml

Table 3.6. Experimental details for ELISAs.

3.8.4 Data analysis

A standard curve was constructed using the Gen5 program (BioTek UK, Swindon) to generate a 4-parameter logistic curve fit, with R^2 >0.98. Apparent concentrations were multiplied by the dilution factor to provide sample concentrations. Mean values with a coefficient of variation (CV) <20% were accepted.

3.9 *Limulus* amoebocyte assay for the detection of lipopolysaccharide

3.9.1 Assay principles

Like the ELISA assay, the *Limulus* amoebocyte lysate (LAL) assay is an enzymatic chromogenic assay, where concentration of LPS is quantified by measuring colour intensity.

Innate immune blood cells (amoebocytes) from the horsehoe crab *Limulus polyphemus* react to LPS *in vivo* by promoting the blood coagulation cascade, with the biological effect of containing LPS-containing bacteria. The proteolytic cascade responsible for this reaction has been modified so that a purified amoebocyte lysate results in the cleavage of the colourless chromophore *para*-nitroanilide to produce *para*-nitroaniline (*p*NA), which is yellow and absorbs at 405 nm, when the lysate is activated by the presence of LPS. The rate of *p*NA production, and therefore the intensity of colour produced, is related to the amount of LPS in the sample, and can be measured by comparing to a standard curve of known concentrations.

3.9.2 Method

Fresh whole blood was centrifuged at 1000*g* for 15 minutes at 4°C. The plasma was then aliquoted and stored at –20°C until required. LAL assays were performed using a Pyrochrome ® kit (Associates of Cape Cod International Inc., Liverpool, UK) according to the manufacturer's instructions. Samples were diluted with LAL reagent-grade water (Associates of Cape Cod International Inc., Liverpool, UK) to give a 1000-fold sample dilution. Controls and standards were prepared using 2-fold serial dilutions using LAL reagent-grade water to provide a reference range between 0 and 5.12 EU/mL. Samples, standards and controls were plated in duplicate. Optical densities were determined immediately after stopping with a microplate reader measuring absorbance at 405nm (EL800, BioTek UK, Swindon).

3.9.3 Analysis

A standard curve was constructed by plotting optical density readings against standard endotoxin concentrations using Gen5 (BioTek UK, Swindon), with R^2 >0.98. Apparent concentrations were multiplied by the dilution factor to provide sample concentrations. Mean values with a coefficient of variation (CV) <20% were accepted.

3.10 Assessment of nutritional status and body composition

A number of assessments of body composition were used before and after the supplementation period.

3.10.1 BMI

Height and weight were measured in the community research clinic, in the NuSTART research clinic, and by air displacement plethysmography (BodPod ®). Correlation between the three measurements was excellent (Table 3.7). Of these, the weight measurements used for BodPod body composition analysis were the most accurate and were therefore used for analysis.

	ρ	***P***
Misisi v. NuSTART	0.973	**<0.001**
Misisi v. BodPod	0.967	**<0.001**
Nustart v. BodPod	0.998	**<0.001**

Table 3.7. Spearman rank correlations between 3 different measurements of BMI.

3.10.2 Grip strength

Dynamometry was performed before the endoscopy procedure, to avoid any effect of sedation. Non-dominant grip strength was measured using a calibrated dynamometer (Takei Scientific Instruments, Niigata, Japan), with participants in a standing position with the arm by their side and elbow extended. The 'best attempt of three' reading was recorded by one of two study nurses.

3.10.3 Mid-upper arm circumference (MUAC)

Participants' non-dominant MUAC was measured using a standardised tape measure using a blinded technique by a single trained research clinic nurse.

3.10.4 Bioelectrical impedance analysis (BIA)

3.10.4.1 Principle (Ellis, 2000)

Impedance has both resistive and reactive (capacitative) components, with resistance deriving from intra- and extracellular fluid and reactance deriving from cell membranes. At a current frequency of 0 (i.e. direct current), reactance increases so that impedance (Z_0) is equal to total body resistance (R_0) which reflects passage of current solely through the extracellular fluid compartment. Conversely, at infinite frequency, cell membranes will behave as perfect capacitors, so that impedance (Z_∞) will be equal to total body resistance (R_∞) and reflect passage of current through both extracellular and intracellular fluid compartments. Because these extremes are theoretical, measured impedance can be used to derive R_0 and R_∞ using Cole-Cole plots, which describe reactance against resistance when frequency and impedance are known.

A weak alternating current at 50kHz is passed through the body through electrodes attached to the subject's hands and feet, and impedance is measured. Because fat has an extremely high impedance, BIA in practice measures impedance in total cellular mass and extracellular water (i.e. fat free tissue). Incorporating

empirically derived constants to correct for age, sex, weight etc. allows fat free mass to be estimated, and by comparing this to measured weight, fat mass.

3.10.4.2 Method

Bioelectrical impedance was measured using a Tanita analyser (Tanita Corporation, Tokyo, Japan) according to the manufacturer's instructions. All measurements were performed while fasting to avoid the confounding factor of fluid intake on total body water estimation.

3.10.5 Air displacement plethysmography

3.10.5.1 Principle (Ellis, 2000)

These models all rely on measurement of total body density. Once this has been determined, a number of methods exist to extrapolate mean (total) body density to various body compartments. Two compartment models divide tissue into fat mass and fat-free mass compartments, using experimentally derived formulae. Cadaveric body density assessment is viewed as the gold standard. Allowance must be made for race-related differences in body composition; of particular note is the increased muscle, bone and mineral density of people of black African descent. For this population the Ortiz (for women) (Ortiz et al., 1992) and Schutte (for men) (Schutte et al., 1984) equations provide the best validated estimates of lean and fat mass.

ADP measures weight using a highly sensitive and atmospherically isolated balance. Body volume is calculated by measuring air pressure within an atmospherically sealed capsule of known volume: by altering the volume by a known amount (by use of a diaphragm) and measuring the change in pressure, body volume can be calculated using Boyle's law ($V_2 = [P_1V_1]/P_2$).

The effect of thoracic (air) volume must also be considered. Total gas volume (TGV) can accurately be determined using exhalational volumetry, although it can

be accurately estimated using experimentally derived equations incorporating height, age and gender (McCrory et al., 1998).

3.10.5.2 Air displacement plethysmography ('BodPod' ®) – method

In many cases participants found it difficult to follow the multi-step breathing commands which were required to accurately assess TGV volumetrically, so predicted TGV was used.

Air displacement plethysmography was conducted using BodPod ® (Cosmed, Rome, Italy) according to the manufacturer's instructions while participants were fasted. Participants were asked to change into slim fitting swimwear and a swim cap to minimise artefactual volume due to clothing and hair.

CHAPTER 4

RESULTS: AMAZE TRIAL OVERVIEW, PRIMARY OUTCOMES, AND SAFETY

4.1 Study participants

The trial was carried out between October 2015 and May 2016. The timing of recruitment was such that treatment was initiated in November – December 2015 (before the rainy season), and the final assessments after treatment were made in March – April 2016 (after the rainy season).

136 healthy adult volunteers were screened, of whom 102 met all inclusion and exclusion criteria and were recruited and randomised. All of these completed the baseline study assessments. 85 participants completed the supplementation period and underwent assessments at the end of the supplementation period. One participant was unable to tolerate the post-intervention endoscopy so samples and data from 84 participants were available for analysis. The CONSORT trial flowchart (Schulz et al., 2010) is given in Figure 4.1. Baseline characteristics of the participants by treatment group at randomisation is given in Table 4.2 (p.140), and in the per-protocol group in Table 4.3 (p.141).

In general, participants were young and female. Participants were significantly stunted (median height between the 3rd and 15th centiles for men, and on the 15th centile for women compared to WHO reference standards (World Health Organization and Onis, 2006). 12.1% of men and 43.5% of women were overweight (BMI >25), whereas no men and 17.4% of women were obese (BMI > 30). In terms of deprivation markers, only 45% had completed primary education, 30% had no household electricity, and only 24% regularly used clean (boiled or chlorinated) water. 44/102 (43.1%) of randomised participants had a total of 83 non-pathogenic parasites identified on stool microscopy (Table 4.1).

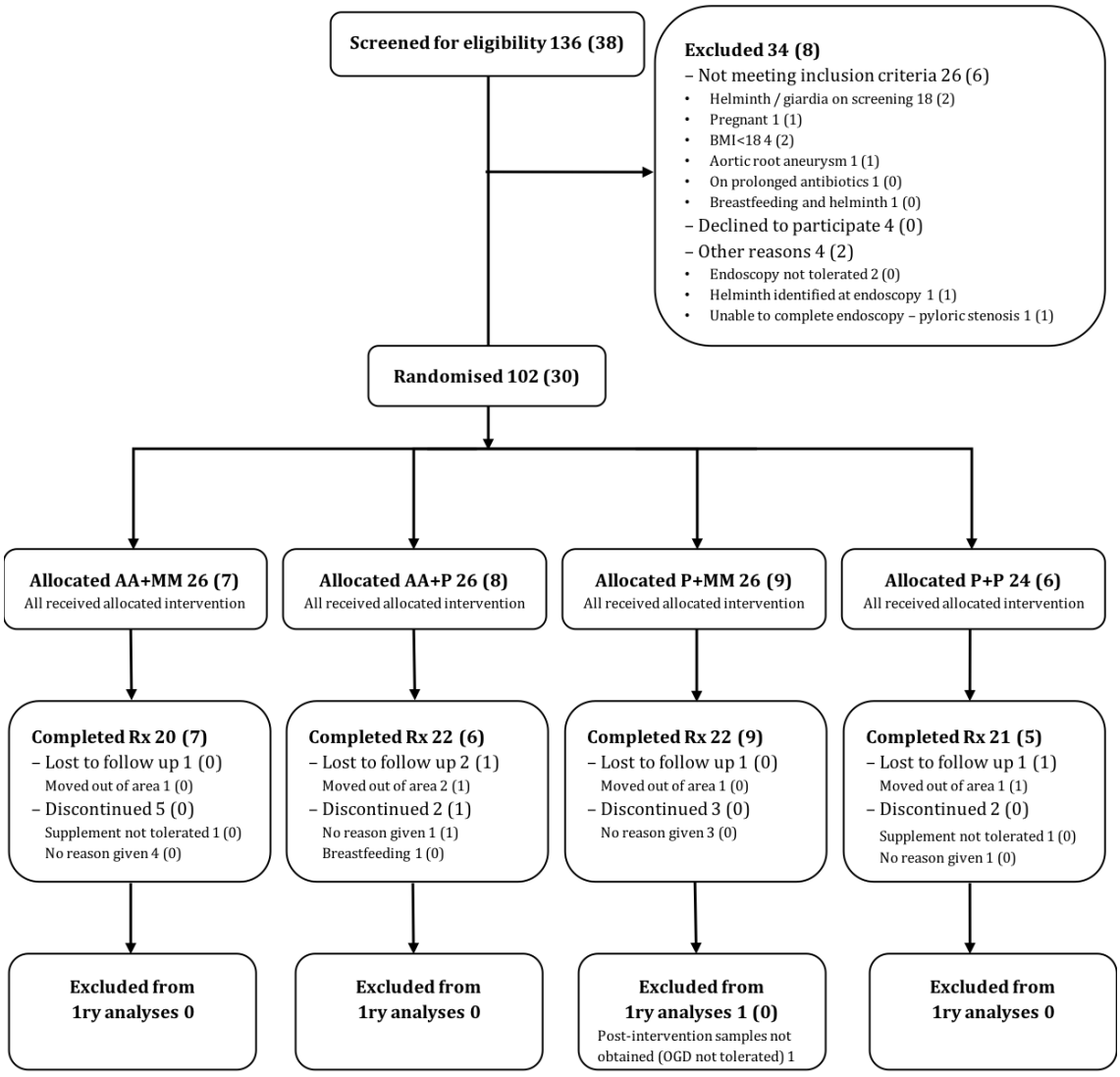

Figure 4.1. CONSORT flow chart. Total numbers of participants are given with numbers of HIV positive participants in brackets.

Parasite	Number of stool samples
Endolimax nana	28
Entamoeba coli	27
Blastocystis hominis	15
Iodamoeba bütschlii	7
Retortamonas intestinalis	5
Entamoeba hartmanni	1
Total	**83**
No parasites identified (% of participants)	**58 (56.9)**
One or more parasites identified (% of participants)	**44 (43.1)**

Table 4.1. Non-pathogenic parasites on stool microscopy in randomised participants.

		Amino acids (n=52)	Placebo (n=50)	Micronutrients (n=52)	Placebo (n=50)
Age, years		32 (22-45)	39 (23-45)	35 (24-45)	34 (22-45)
Sex, M:F		19:33	14:36	17:35	16:34
HIV positive, n (%)		15 (29)	15 (30)	16 (31)	14 (28)
Peripheral CD4 count		548 (305-613)	554 (417-790)	472 (327-697)	582 (467-740)
Height, cm	*Male*	168 (165 – 174)	168 (164 – 172)	166 (165 – 174)	171 (165 – 173)
	Female	157 (153 – 161)	158 (154 – 161)	157 (153 – 161)	157 (154 – 161)
Body mass index, kg.m^{-2}	*Male*	19.9 (19.0 – 20.2)	20.6 (19.6 – 25.7)	20.1 (19.1 – 23.3)	19.8 (19.1 – 20.5)
	Female	24.6 (21.2 – 25.8)	25.0 (20.7 – 29.0)	23.5 (20.2 – 26.9)	25.1 (20.5 – 28.3)
Body fat by ADP, %	*Male*	16.9 (14.7 – 19.0)	11.4 (7.8 – 25.8)	14.3 (10.7 – 18.3)	14.8 (11.0 – 17.7)
	Female	33.7 (24.5 – 35.8)	36.1 (27.6 – 44.1)	34.0 (24.1 – 40.5)	34.3 (27.7 – 39.3)
Grip strength, kg	*Male*	39.5 (36.2 – 41.9)	39.1 (36.1 – 42.2)	40.8 (34.9 – 45.8)	39.1 (36.2 – 41.6)
	Female	27.2 (23.4 – 31.3)	28.1 (25.9 – 33.0)	28.6 (26.4 – 34.7)	26.9 (24.2 – 32.2)
Completed primary education, %		36	54	42	48
Household Hygiene Score [a], (0-10)		6 (5-7)	6 (5-7)	6 (5-7)	6 (5-7)
Household electricity, %		71.2	66	69.2	68
Routinely boil water [b], %		7.6	6	5.7	8
Routinely chlorinate water [b], %		17.3	30	19.2	28
Non-pathogenic stool parasites, %		48.1	38	46.2	40

Table 4.2. Baseline characteristics of the study participants at randomisation by treatment group. Values given as median (IQR) where applicable. ADP, air displacement plethysmography; BMI, body mass index. [a], the Household Hygiene Score is a reproducible objective measure of the living conditions of each participant and ranges from 0 (worst) to 10 (best). Trained fieldworkers score up to 2 points in each of five categories (overall cleanliness; water storage facilities; food storage facilities; hand washing facilities and their use; sanitation facilities). [b], defined as most or all days.

		Amino acids (n=42)	Placebo (n=42)	Micronutrients (n=41)	Placebo (n=43)
Age, years		35 (28-46)	42 (24-51)	40 (21-30)	40 (30-46)
Sex, M:F		16:26	10:32	11:30	15:28
HIV seropositive, n (%)		13 (31)	14 (33)	16 (38)	11 (26)
Peripheral CD4 count		510 (268 – 683)	621 (422 – 800)	472 (305 – 750)	602 (387 – 827)
Height, cm	*Male*	169 (166 – 174)	171 (165 – 172)	166 (165 – 173)	171 (166 – 174)
	Female	157 (154 – 162)	158 (154 – 161)	157 (153 – 161)	157 (154 – 161)
Body mass index, kg.m^{-2}	*Male*	19.9 (19.0 – 20.2)	20.6 (19.6 – 25.7)	20.6 (20.0 – 25.4)	19.7 (19.1 – 20.2)
	Female	24.6 (21.2 – 25.8)	25.0 (20.7 – 29.0)	23.5 (20.1 – 27.4)	25.1 (21.4 – 27.9)
Body fat by ADP, %	*Male*	16.9 (14.7 – 19.0)	11.4 (7.8 – 25.8)	17.5 (11.6 – 24.1)	15.8 (11.0 – 18.6)
	Female	33.7 (24.5 – 35.8)	36.1 (27.6 – 44.1)	34.3 (23.9 – 40.2)	34.5 (27.9 – 39.0)
Grip strength, kg	*Male*	39.5 (36.2 – 41.9)	39.1 (36.1 – 42.2)	40.2 (35.7 – 46.6)	39.2 (36.4 – 41.7)
	Female	27.2 (23.4 – 31.3)	28.1 (25.9 – 33.0)	28.7 (26.5 – 34.0)	26.7 (23.6 – 31.6)
Completed primary education, %		35.7	51.2	40.5	46.5
Household hygiene score [a], 0-10		6 (5 – 7)	6 (5 – 6)	6 (5 – 6.5)	5 (5 – 8)
Household electricity, %		69.1	65.1	69.1	65.1
Routinely boil water [b], %		9.5	4.7	7.0	7.1
Routinely chlorinate water [b], %		14.3	34.9	19.1	30.2
Non-pathogenic stool parasites, %		47.6	39.5	42.9	44.2

Table 4.3. Baseline characteristics in per-protocol participants. See Table 4.2 for notes and explanations.

4.1.1 Compliance

Compliance as assessed by monthly supplement counts was high: median (IQR) percentage of amino acid supplement packs consumed was 73 (60-86)% and of micronutrient capsules taken was 80 (67-88)%. Adjustment for the proportion of supplement consumed in a linear regression model made no difference to the results presented below. The correlation between MM and AA consumption was high (ρ 0.85, $P < 0.001$).

Reasons for supplement discontinuation and study withdrawal, where given, are provided in Table 4.4 and Figure 4.1.

	Number of participants
Lost to follow up (moved out of area)	5
Supplement not tolerated	2
No reason given	9
Breastfeeding	1
Total	**17**

Table 4.4. Reasons for withdrawal from the study.

4.2 Effect of AA or MM supplementation on proximal small intestinal absorptive area

As in previous studies from this population, participants exhibited histological evidence of severe enteropathy at baseline, with marked villous atrophy (median villus height 200.2μm, IQR 167.5μm – 242.1μm) and dramatically reduced villus height to crypt depth ratio (median 1.3, IQR 1.0 – 1.4) (Figure 4.2).

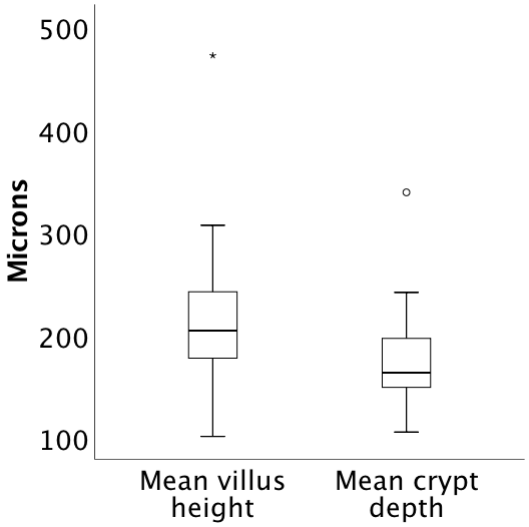

Figure 4.2. Marked villus atrophy and crypt hypertrophy at baseline in study participants.

	AA (n=26)	**Placebo (n=23)**	**MM (n=26)**	**Placebo (n=23)**
VH (µm)	185 (154-217)	218 (201-250)	202 (181-252)	206 (170-235)
CD (µm)	163 (141-196)	163 (150-198)	157 (135-194)	174 (152-199)
VW (µm)	230 (210-263)	222 (197-268)	221 (202-253)	230 205-267)
VP (µm) per 100µm muscularis mucosae	865 (702-1030)	987 (868-1134)	931 (788-1072)	913 (772-1090)
VA (µm²) per 100µm muscularis mucosae	30061 (23036-37178)	32328 (27992-40093)	31998 (23619-37642)	31288 (24892-37200)

Table 4.5. Mean baseline morphometric parameters by intervention group. VH, villus height; CD, crypt depth; VW, villus width; VP, villus perimeter; VA, villus cross-sectional area.

There was a general reduction in villus and crypt measurements between the first and second biopsies (Table 4.6 and Table 4.7), taken before and at the end of the rainy season. The reduction in villus height and villus cross-sectional area was negated in those participants receiving amino acid supplementation compared to placebo (Table 4.6 and Figure 4.3, p.145). Micronutrient supplementation had no effect on any morphometric measurement (Table 4.7 and Figure 4.4, p.145).

Change in:	AA (n=26)	Placebo (n=23)	*P*
Villus Height (µm)	2.5 (59)	-32.0 (57)	**0.04**
Crypt Depth (µm)	-13.3 (39)	-31.1 (45)	0.2
Villus Width (µm)	-11.5 (60)	-13.7 (138)	0.9
Villus Perimeter (µm) per 100 µm muscularis mucosae	-1.9 (180)	-89.5 (159)	0.08
Villus Area (μm^2) per 100 µm muscularis mucosae	715 (5632)	-3220 (5694)	**0.02**
Villus Surface Area:Volume Ratio ($x10^{-4}$)	-9 (7)	3 (60)	0.5

Table 4.6. Effect of AA supplementation on change in morphometric parameters over the supplementation period. Independent samples t test; values given as mean (SD).

Change in:	MM (n=26)	Placebo (n=23)	*P*
Villus Height (µm)	-19.5 (62)	-8.6 (60)	0.5
Crypt Depth (µm)	-25.5 (43)	-18.3 (43)	0.6
Villus Width (µm)	-16.6 (72)	-8.9 (126)	0.8
Villus Perimeter (µm) per 100 µm muscularis mucosae	-64.1 (137)	-24.4 (203)	0.4
Villus Area (μm^2) per 100 µm muscularis mucosae	-2469 (5061)	51 (6494)	0.1
Villus Surface Area:Volume Ratio ($x10^{-4}$)	-0.4 (60)	-6 (60)	0.8

Table 4.7. Effect of MM supplementation on change in morphometric parameters over the supplementation period. Independent samples t-test; values given as mean (SD).

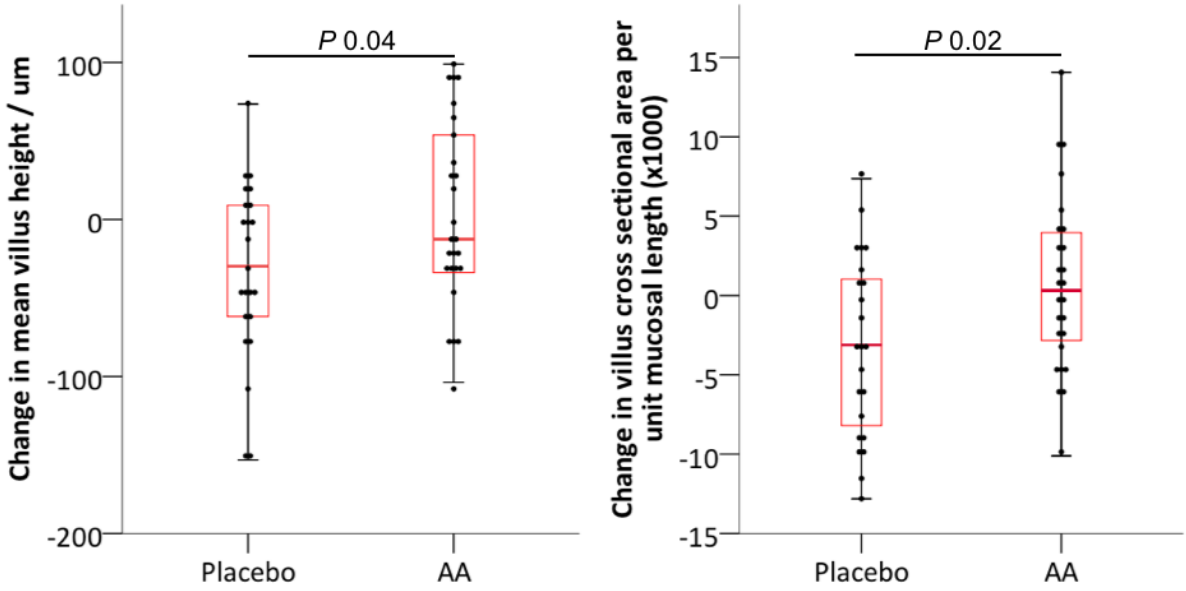

Figure 4.3. Effect of AA supplementation on change in morphometric parameters over the supplementation period. Independent samples t-test.

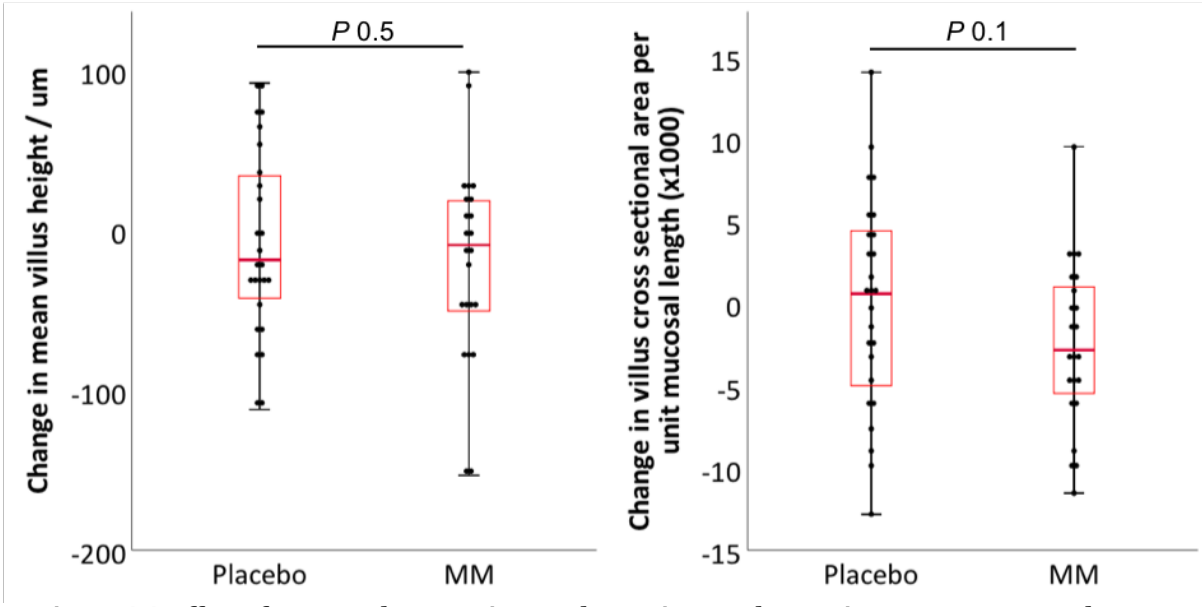

Figure 4.4. Effect of MM supplementation on change in morphometric parameters over the supplementation period. Independent samples t-test.

Examining for main effects and interactions by univariate ANOVA confirmed a significant effect of amino acid supplementation on the change in VH and VA, such that participants who received AA were protected against the seasonal decline compared to those who received placebo (*P* 0.05 for VH; *P* 0.027 for VA). There was no evidence of an MM supplementation main effect (*P* 0.65 for VH; *P* 0.2 for VA) or of an interaction between AA and MM (*P* 0.7 for VH; *P* 0.9 for VA).

4.3 Effect of amino acid or multiple micronutrient supplementation on small intestinal barrier dysfunction as assessed by CLE

The number of endomicroscopic fields demonstrating pathological barrier dysfunction (one or more of erosions; fluorescein plumes; intraluminal fluorescein; intramucosal fluorescein) was assessed by two independent blinded observers. Images of adequate quality were available in 81/85 patients before supplementation and 77/84 after supplementation. The median (IQR) number of fields available for analysis was 72 (49-98) at baseline and 98 (78-117.5) after supplementation. Images were rated by two observers; inter-observer correlation was high (ρ=0.94; *P*<0.001; Figure 4.5).

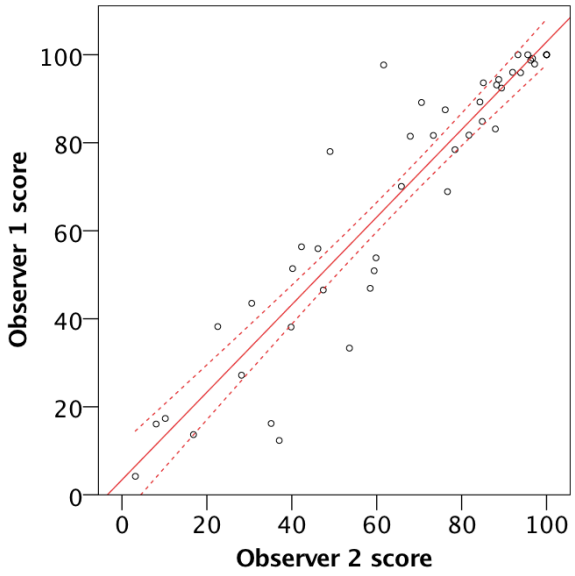

Figure 4.5. Interobserver correlation between two independent blinded assessors of pathological endomicroscopic barrier defects.

Levels of barrier dysfunction were very high in all groups at baseline (Table 4.8; Figure 4.6), and did not change over the course of the study. 71/81 (87.7%) of participants at baseline and 65/77 (84.4%) of participants post-intervention demonstrated barrier dysfunction in more than 30% of fields.

	AA	**Placebo**	**MM**	**Placebo**
Median (IQR) fields with dysfunction, %	79.1 (57.2-88.5)	85.8 (75.9-91.6)	80.5 (57.2-91.1)	86.0 (75.0-91.6)

Table 4.8. Endomicroscopic barrier dysfunction at baseline.

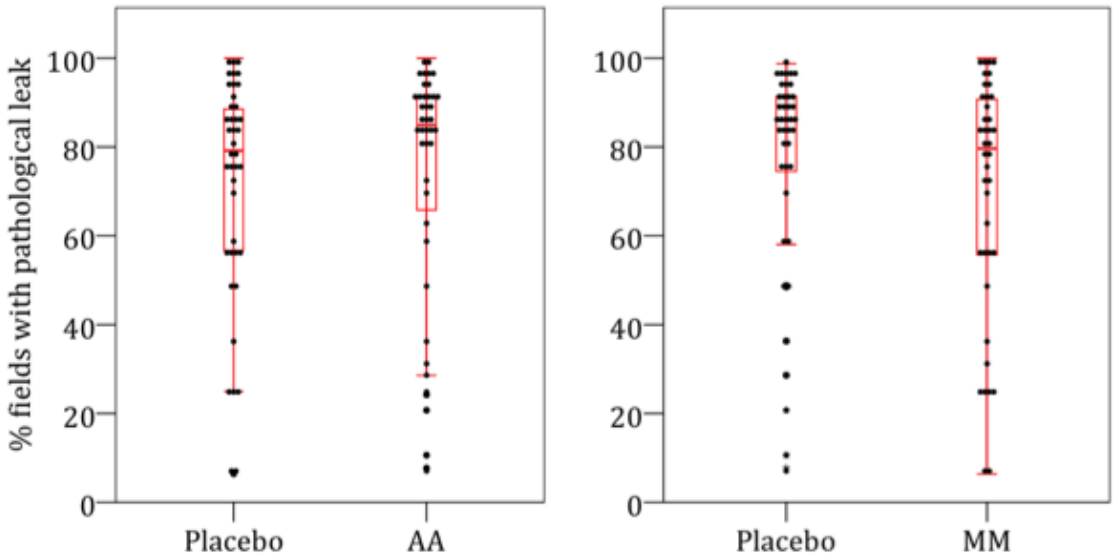

Figure 4.6. Baseline endomicroscopic barrier dysfunction by treatment group. Left, AA v. placebo. Right, MM v. placebo.

Neither intervention altered levels of leak observed (Figure 4.7). Examining for main effects and interactions by univariate ANOVA confirmed there was no evidence of a main effect of AA or MM supplementation on change in endomicroscopic leak (*P* 0.35 for AA; *P* 0.31 for MM). However, there was evidence of a significant interaction (*P* 0.004) with a crossover effect: a significant reduction in endomicroscopic leak was observed in participants who received both AA and MM supplements (Figure 4.8).

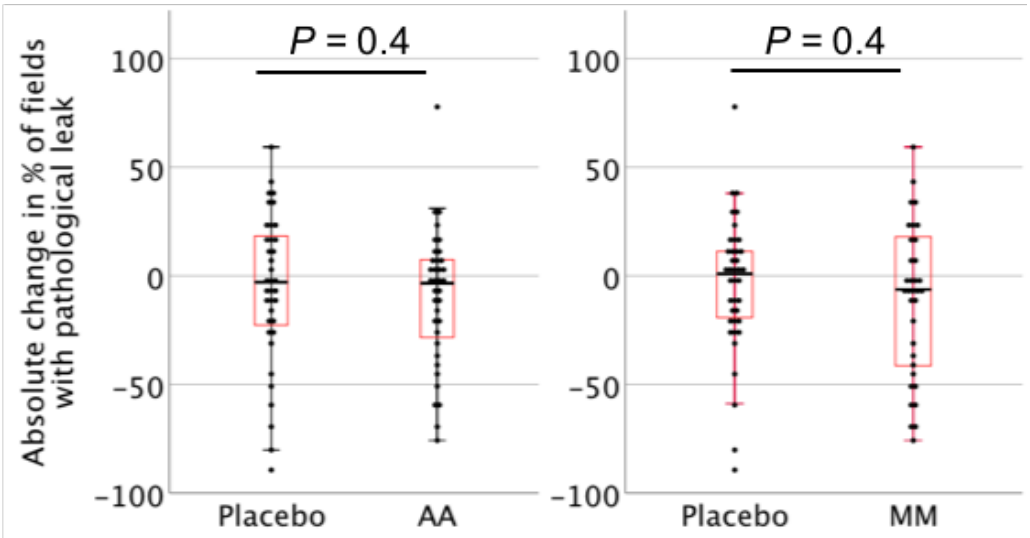

Figure 4.7. Change in endomicroscopic barrier dysfunction by intervention group.

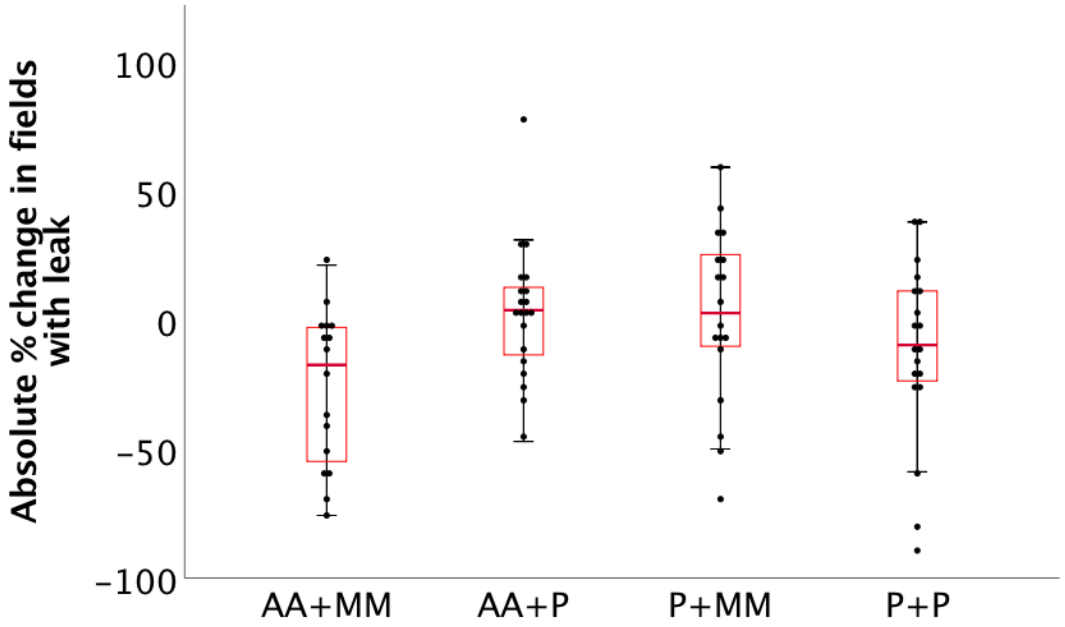

Figure 4.8. Interaction effect of supplements on change in endomicroscopic barrier dysfunction. P = 0.023 across all groups (one-way ANOVA).

4.4 Effect of AA or MM supplementation on mucosal T lymphocyte MTORC1 activity

The responsiveness of lamina propria T lymphocytes was assessed by measuring the proportion of phospho-4EBP1 positive cells following *in vitro* nutrient stimulation. Approximately 50% of lamina propria $CD4^+$ T cells were nutrient responsive (phospho-4EBP1 positive) at baseline (Table 4.9). $CD4^+$ basal MTORC1 activity (measured as phospho-4EBP1 MFI after nutrient deprivation) and maximal MTORC1 activity (phospho-4EBP1 MFI after nutrient stimulation) declined over the supplementation period in all four arms (Figure 4.9, p.150). The proportion of nutrient responsive (phospho-4EBP1 positive) $CD4^+$ T cells was unchanged in all arms apart from participants who received MM, where the proportion of responsive cells declined (Figure 4.9). Neither intervention altered nutrient responsiveness or basal or maximal MTORC1 activity compared to placebo (Table 4.10 and Figure 4.10, p.151).

	AA (n=14)	Placebo (n=23)	MM (n=18)	Placebo (n=19)
% p4EBP1 positive	47.9 (41.1 – 58.3)	60.0 (44.6 – 67.0)	58.1 (46.8 – 65.1)	48.3 (32.9 – 61.9)

Table 4.9. Proportion of CD3⁺ CD4⁺ lamina propria lymphocytes responding to in vitro nutrient stimulation at baseline.

Change in:	AA	Placebo	*P*	MM	Placebo	*P*
Basal p4EBP1 MFI	-152 (56)	-125 (85)	1.0	-121 (67)	-150 (81)	0.3
Max. p4EBP1 MFI	-288 (128)	-276 (126)	0.8	-281 (114)	-282 (138)	0.9
% p4EBP1 positive	-9.0 (18.5)	-10.2 (19.1)	0.9	-15.0 (13.5)	-4.8 (21.5)	0.2

Table 4.10. Change in lamina propria CD4⁺ MTORC1 signalling according to intervention.

Examining for main effects and interactions by univariate ANOVA confirmed there were no main or interaction effects for the interventions on the change in phospho-4EBP1 positivity (*P* 0.6 for AA; *P* 0.14 for MM; *P* 0.5 for interaction), basal MTORC1 activation (*P* 0.3 for AA; *P* 0.3 for MM; *P* 0.5 for interaction), or maximal MTORC1 activation (*P* 0.8 for AA; *P* 0.9 for MM; *P* 0.4 for interaction). The Shapiro-Wilk test confirmed normality for these outcomes.

The proportion of MTORC1 responsive cells was correlated with maximal MTORC1 activity, but not with basal activity. Basal and maximal MTORC1 activity was positively correlated (Table 4.11). These correlations were stronger and more significant across all treatment groups as a whole after the intervention period (Table 4.11; overall correlations across treatment groups shown).

	Pre-supplementation		Post-supplementation	
	ρ	***P***	**ρ**	***P***
% responsive v. basal activity	-0.2	0.3	0.1	0.6
% responsive v. max. activity	0.4	**0.01**	0.5	**0.001**
Basal v. max. activity	0.6	**0.001**	0.9	**<0.001**

Table 4.11. Correlations between three different measurements of MTORC1 activity.

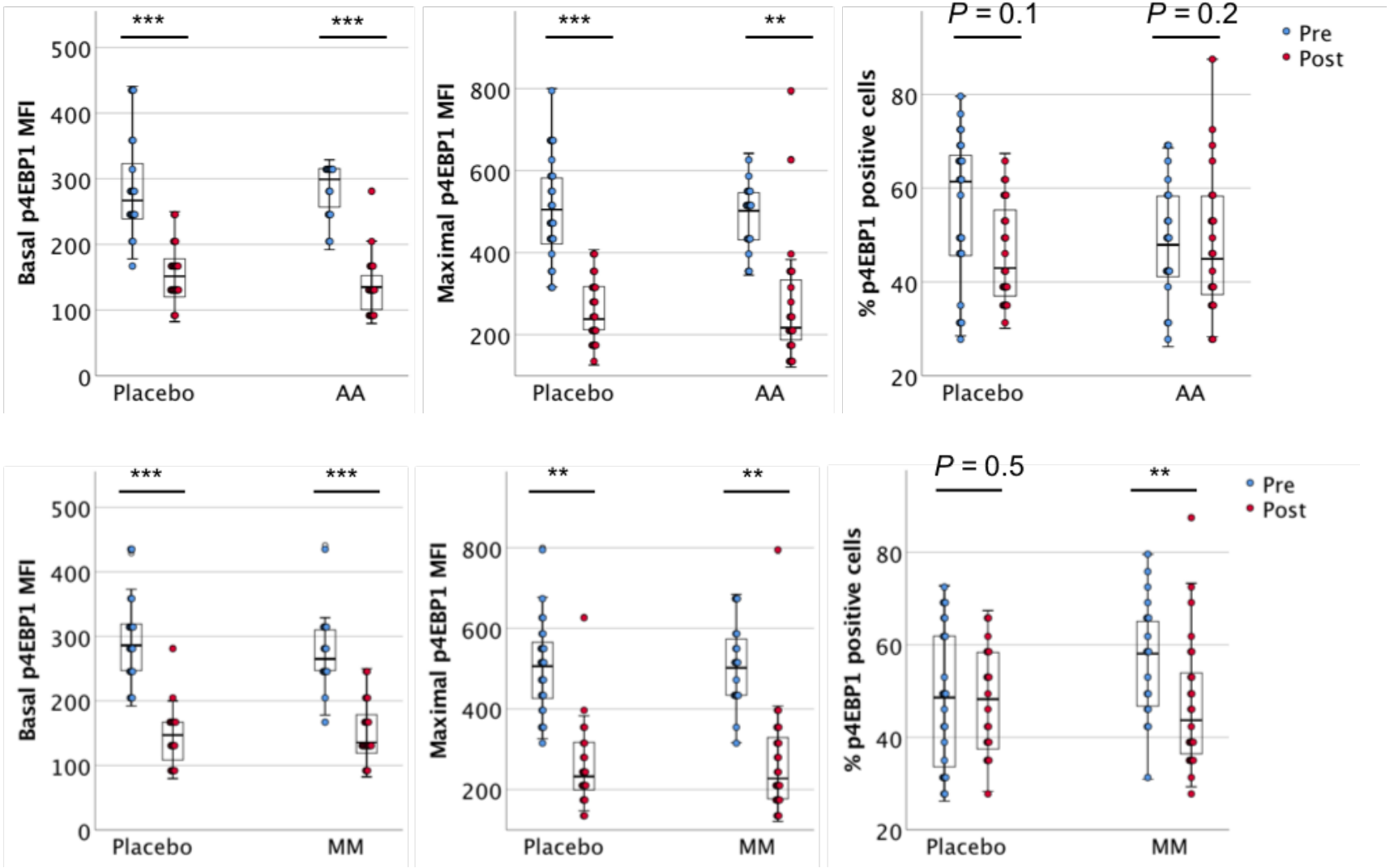

Figure 4.9. LP CD4⁺ MTORC1 activity before & after the supplementation period, showing basal, maximal & % responsive. Top, AA v. placebo; bottom, MM v placebo.

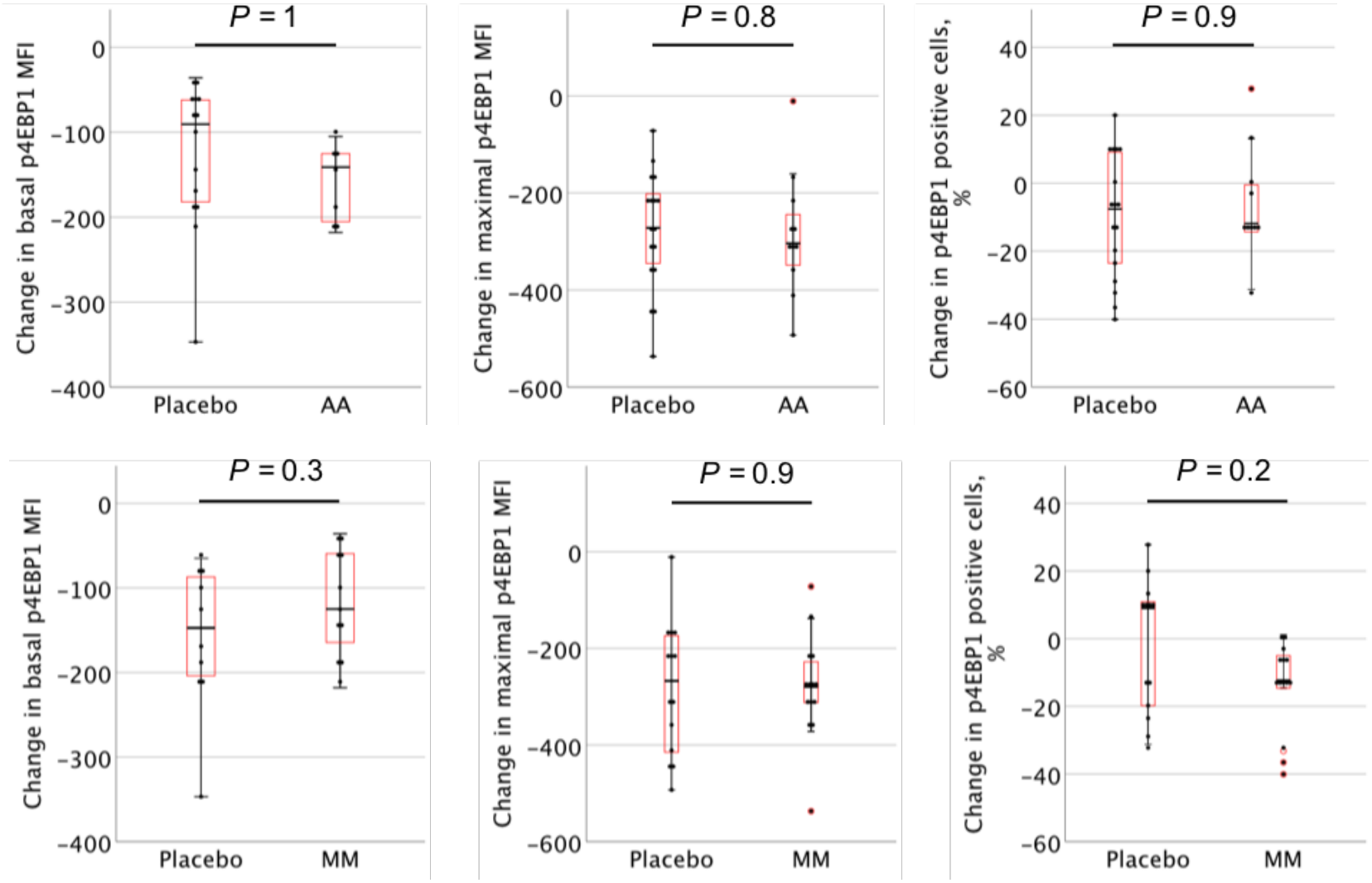

Figure 4.10. Effect of supplementation on change in LP CD4⁺ MTORC1 activity. Top, AA v. placebo. Bottom, MM v. placebo.

Although MTORC1 responsiveness was not affected by treatment allocation, it was correlated with post-treatment VH in both AA and MM groups (ρ = 0.56; *P* = 0.04 and ρ = 0.53; *P* = 0.01 respectively; Figure 4.11), but not their corresponding placebos (ρ = 0.4; *P* = 0.2 and ρ = 0.4; *P* = 0.1 for AA placebo and MM placebo, respectively). However there was no correlation at baseline, and the change in VH was not correlated to baseline MTORC1 activity (data not shown).

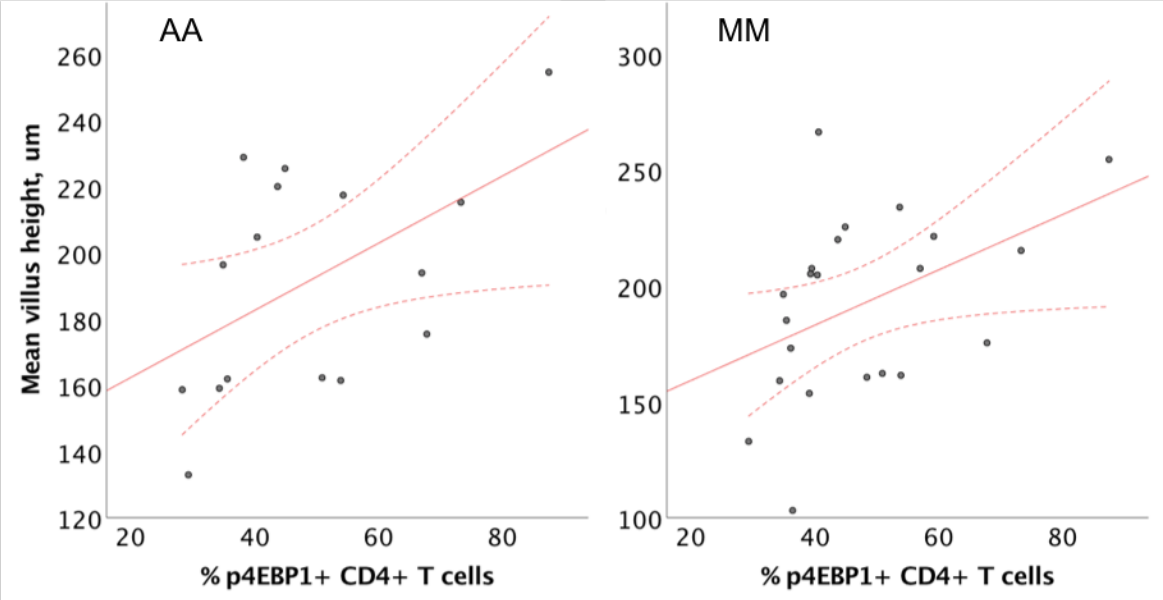

Figure 4.11. Correlation between MTORC1 responsiveness and villus height after the supplementation period in active treatment groups.

4.5 Effect of HIV status

4.5.1.1 Baseline characteristics in per-protocol population

The HIV seropositivity prevalence at randomisation was 29.4% (30/102 participants), reflecting the known HIV prevalence of 30% in this population. 5/30 were new diagnoses. 20/25 of established diagnoses were on HAART, with a median duration of therapy of 7 years (IQR 2.75 – 9 years). Peripheral $CD4^+$ counts were robust (median 550, IQR 372-720, range 129 – 999), and only 2 participants had a $CD4^+$ count of <200.

HIV positive men were under-represented in the per-protocol population. HIV positive participants were also older. Interestingly, non-pathogenic stool parasites were less prevalent in HIV positive participants (Table 4.12).

		HIV negative (n=57)	HIV positive (n=27)	*P*
Age		34.4 (22.0 – 47.0)	41.0 (35.0 – 50.0)	**0.02**
Sex, M:F		22:35	4:23	**0.02** [a]
Height, cm	*Male*	167 (165 – 172)	173 (171 – 174)	0.08
	Female	156 (153 – 161)	159 (156 – 162)	0.09
Body mass index, kg.m^{-2}	*Male*	20.1 (19.2 – 21.2)	19.9 (19.3 – 24.4)	0.8
	Female	25.0 (22.2 – 28.4)	23.3 (20.0 – 27.6)	0.08
Fat % by ADP	*Male*	15.9 (11.0 – 18.0)	20.0 (14.5 – 30.0)	0.2
	Female	34.2 (27.7 – 41.1)	34.3 (24.3 – 38.8)	0.3
Grip strength, kg	*Male*	39.4 (36.2 – 42.7)	38.0 (34.8 – 41.3)	0.5
	Female	27.6 (24.2 – 33.2)	28.3 (24.2 – 30.5)	0.9
Completed primary education, %		44.8	40.7	0.7 [a]
Household hygiene score, 0-10		6 (5 – 7)	6 (5 – 6)	0.15
Household electricity, %		72.4	55.6	0.1 [b]
Routinely boil water		2/57	4/27	0.08 [a]
Routinely chlorinate water		14/57	7/27	1 [a]
Non-pathogenic stool parasites, %		53.4	22.2	**0.007** [b]

Table 4.12. Baseline characteristics in HIV positive and negative participants Values are given as median (IQR) unless otherwise stated. For explanatory notes see Table 4.2 (p. 140). P values derived using Mann-Whitney U test unless otherwise stated. [a] Fisher's exact test. [b] χ^2 test.

4.5.1.2 Effect of HIV on primary outcomes

There were no differences in baseline parameters between HIV positive and negative participants (Table 4.13).

	HIV negative		HIV positive		*P*
	n		n		
VH, µm	42	200 (168 – 242)	19	212 (190 – 249)	0.5
CD, µm	42	166 (139 – 197)	19	163 (152 – 199)	0.9
VW, µm	42	229 (204 – 256)	19	223 (199 – 289)	1
VA per unit MM	42	17900 (13600 – 21200)	19	18300 (15400 – 21300)	0.9
VP per unit MM	42	511 (423 – 676)	19	512 (457 – 590)	1
% $CD4^+$ $p4EBP1^+$	33	49.5 (39.7 – 65.0)	11	57.1 (48.6 – 77.4)	0.4
Basal p4EBP1 MFI	33	265 (239 – 318)	11	300 (261 – 349)	0.4
Max. p4EBP1 MFI	33	502 (434 – 563)	11	510 (394 – 597)	1
% fields with leak	54	83.2 (56.7 – 91.3)	27	83.3 (62.8 – 91.7)	0.9

Table 4.13. Baseline parameters in HIV positive and negative participants.

No significant differences between HIV seropositive and seronegative participants were observed on the primary outcomes when analysed by treatment group; however in many cases too few paired samples were available for HIV positive participants to draw meaningful conclusions. Furthermore, no main or interaction effects were observed between HIV status and either or both of the interventions, and a regression model (section 4.6) also confirmed that HIV was not contributing.

Although the proportion of nutrient responsive $CD4^+$ T cells was similar at baseline between HIV positive and negative participants, HIV positive participants who received AA supplementation had a significantly higher proportion of MTORC-responsive $CD4^+$ T cells compared to placebo (Figure 4.12). Due to the small numbers of participants with available paired samples, it could not be shown that AA supplementation affected a change in phospho-4EBP1 positivity or activity in HIV positive participants.

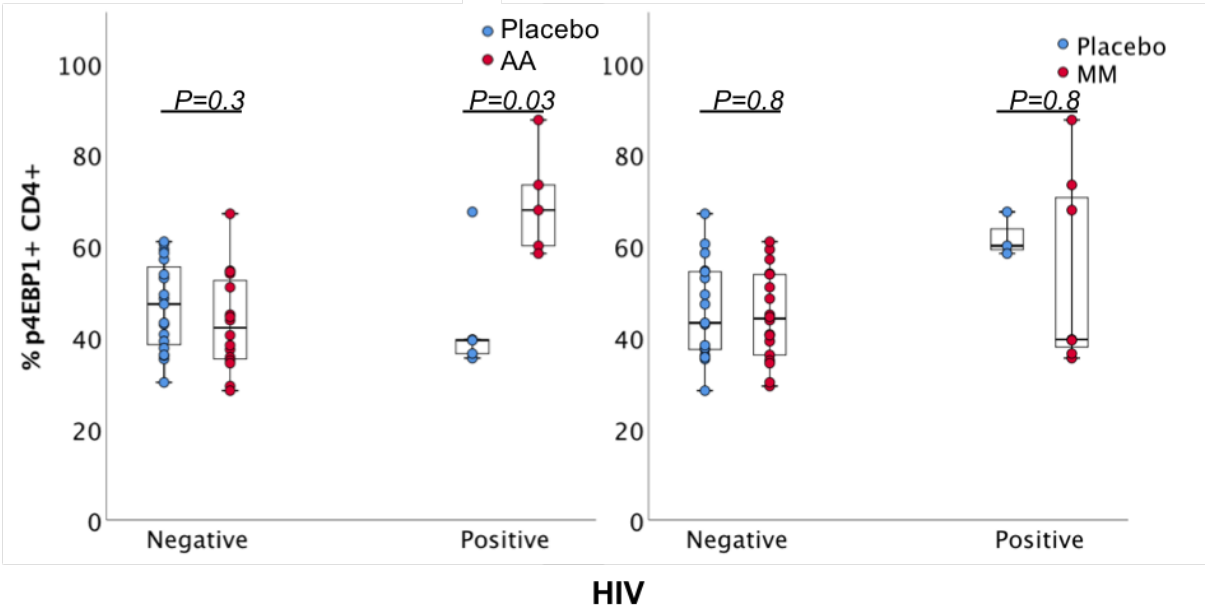

Figure 4.12. Effect of AA supplementation on lamina propria CD4⁺ T cell nutrient responsiveness post-intervention.

4.6 Correlation between small intestinal structure, function and lamina propria lymphocyte nutrient responsiveness

There was no correlation between baseline morphometry, MTORC1 activity, and endomicroscopic barrier dysfunction (Table 4.14). Furthermore, no correlations were observed between any of the primary outcomes. In particular, the change in VH, fluorescein leak, and CD4⁺ T cell MTORC1 activity were not associated with each other, either over all or by treatment group (Table 4.15; overall correlations shown). In a multivariate linear regression model examining the effect of supplement type, HIV status, lean body mass percentage at baseline (as a global marker of health) and endomicroscopic leak at baseline on change in villus height, only AA supplementation emerged as a statistically significant predictor (*P* 0.04), although the model had poor predictive value (adjusted R^2 0.04).

Similarly, post-supplementation MTORC1 activity was correlated with post-supplementation VH, and a multivariate linear regression model examining the

effect of HIV status, treatment group, post-supplementation endomicroscopic leak, post-supplementation lean body mass percentage, and post-supplementation proportion of phospho-4EBP1 positive $CD4^+$ T cells, only MTORC1 responsiveness was a significant predictor (*P* 0.046), although again the model explained only a tiny fraction of the variance observed (adjusted R^2 0.01).

	ρ	*P*
VH v. leak	-0.1	0.6
VH v. MTORC1	0.1	0.4
Leak v. MTORC1	-0.2	0.3

Table 4.14. Correlations between baseline parameters.

	ρ	*P*
Change in VH v. change in leak	-0.1	0.5
Change in VH v. change in MTORC1	0.1	0.9
Change in leak v. change in MTORC1	-0.3	0.2

Table 4.15. Correlations between primary outcomes.

4.7 Safety and adverse events

All supplements were well tolerated. The most frequent adverse events were feeling hungry, cough, diarrhoea, nausea, vomiting, and dizziness. There were no serious adverse events and no deaths occurred. Adverse experiences were not more frequent in the amino acid or micronutrient groups (Table 4.16).

Adverse event	Placebo	AA	Placebo	MM
Abdominal fullness		1	1	
Abdominal pain	8	3	4	7
Accidental ingestion of sachet		1	1	
Anorexia		2	1	1
Backache	5	4	5	4
Body pains	3	1	2	2
Body weakness		3		3
Chest pain	1			1
Cough	4	1	2	3
Cough requiring oral antibiotics	6	6	4	8
Diarrhoea (severe)*	5	2	2	5
Diarrhoea (uncomplicated)	5	8	9	4
Dizziness	5	2	3	4
Dysmenorrhoea		1		1
Febrile illness requiring oral antibiotics	1	1		2
Feet swelling	1		1	
Fever	1	1	1	1
Headache	10	13	8	15
Heart palpitations	2		1	1
Herpes zoster		1		1
Hunger	6	15	8	13
Incomplete spontaneous abortion	1		1	
Joint pains	4	3	4	3
Malaise	1			1
Malaria (self-diagnosed)		1	1	
Malarial illness**	3	6	5	4
Nausea	5	10	6	9
Odynophagia		1	1	
Oversedation at endoscopy***	1		1	
Painful legs	2	3	3	2
Phlebitis at sedative injection site	2	1	1	2
Prolonged menstrual bleeding	1		1	
Rash	1			1
Reduced mobility	1			1
Sleepiness		2		2
Sneezing	2	4	2	4
Sore mouth	1		1	
Sore throat	1			1
Sore throat requiring oral antibiotics	1	1	1	1
Toothache		1	1	
Toothache requiring oral antibiotics	1			1
Borborygmi		1	1	
Vomiting	6	6	5	7
Weakness	1	1	1	1
Weight loss		1		1
TOTAL	**98**	**108**	**89**	**117**

Table 4.16. Adverse events. *Diarrhoea classed as severe if required time off work / usual activities, lasted 3 or more days, was bloody, or required medical attention. None of the cases classed as severe were because of dysentery or requiring medical attention. ** There were no confirmed cases of malaria. ***Reversal agents not required.

CHAPTER 5

RESULTS: SECONDARY OUTCOMES

5.1 Lamina propria T_{H1} cells and the role of MTORC1 signalling

The proportion of lamina propria T_{H1} ($CD3^+$ $CD4^+$ $TBET^+$) cells was assessed after the supplementation period and compared across treatment groups.

There was a high proportion of lamina propria T_{H1} lymphocytes (median (IQR)% 81.9 [73.5-76.5]). 29.9 (1-38.4)% of T_{H1} cells had an activated phenotype (HLA-DR^+). Although in HIV positive participants the absolute number of $CD4^+$ LP lymphocytes was reduced (section 5.4, p.162), the proportion of T_{H1} cells (of total LP $CD4^+$ lymphocytes) was not significantly affected by HIV status. The proportion of T_{H1} cells that was activated was also unaffected by HIV status (data not shown).

In HIV negative but not HIV positive participants there was a moderate overall correlation between post-supplementation MTORC1 activity and the proportion of LP T_{H1} cells (ρ 0.58, *P* 0.001 for basal activity, ρ 0.52, *P* 0.002 for maximal activity; ρ 0.3, *P* 0.09 for the proportion of phospho-4EBP1 positive cells; Figure 5.1). However, none of the interventions resulted in a change in the proportion of $CD4^+$ HLA-DR^+ cells (29.4% placebo v. 29.8% AA, *P* 0.8; 32.2% placebo v. 29.2% MM, *P* 0.9).

The change in MTORC1 activity over the supplementation period was not correlated with the proportion of T_{H1} cells after supplementation (ρ 0.1, *P* 0.7 for proportion of phospho-4EBP1 positive cells; ρ 0.1, *P* 0.6 for maximal activity; ρ 0.4, *P* 0.1 for basal activity).

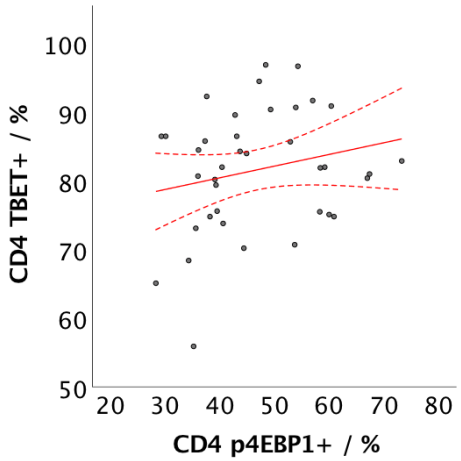

Figure 5.1. Correlation between LP CD4⁺ lymphocyte MTORC1 responsiveness and the proportion of LP CD4⁺ T_{H1} lymphocytes (of all CD4⁺ lymphocytes) in HIV negative participants.

5.2 Correlation between T cell type and activation status on MTORC1 activity

MTORC1 activity in lamina propria T lymphocytes was assessed by flow cytometry using three measures: basal activity (phospho-4EBP1 MFI after nutrient deprivation); maximal activity (phospho-4EBP1 MFI after nutrient stimulation); and percentage phospho-4EBP1 positive. Basal MTORC1 activity was greater in CD8⁺ cells compared to CD4⁺ cells (Table 5.1). Maximal phospho-4EBP1 activity and the proportion of phospho-4EBP1 positive cells was similar in CD4⁺ and CD8⁺ cells. There were no significant differences between HIV positive and negative participants Table 5.2.

	CD4⁺	**CD8⁺**	***P***
Basal p4EBP1 MFI	281 (241 – 318)	290 (241 – 343)	**0.011**
Max. p4EBP1 MFI	502 (424 – 571)	550 (408 – 630)	0.2
% p4EBP1 positive	51.2 (41.6 – 64.8)	46.9 (39.0 – 61.0)	0.2

Table 5.1. MTORC1 activity according to T lymphocyte type.

	HIV negative	HIV positive	*P*
Basal CD4⁺ p4EBP1 MFI	265 (239 – 318)	300 (261 – 349)	0.4
Basal CD8⁺ p4EBP1 MFI	298 (242 – 364)	273 (228 – 325)	0.5
Max. CD4⁺ p4EBP1 MFI	502 (434 – 563)	510 (394 – 597)	0.9
Max. CD8⁺ p4EBP1 MFI	559 (421 – 617)	491 (385 – 648)	0.4
CD4⁺ p4EBP1⁺ %	49.5 (39.7 – 65.0)	57.1 (48.6 – 64.8)	0.4
CD8⁺ p4EBP1⁺ %	49.9 (39.5 – 61.2)	45.0 (36.7 – 56.9)	0.5

Table 5.2. MTORC1 activity markers in CD4⁺ compared to CD8⁺ cells, according to HIV status.

Activated (HLA-DR⁺) CD4⁺ lymphocytes had greater maximal MTORC1 activity compared to resting (HLA-DR⁻) cells (Table 5.3). There were no significant differences in CD8⁺ cells overall. However, when analysed by HIV status, maximal phospho-4EBP1 levels were higher in activated compared to resting CD4⁺ and CD8⁺ T cells in HIV negative participants, and in CD4⁺ but not CD8⁺ T cells in HIV positive participants (Table 5.4). There were no significant differences between HIV positive and negative participants (data not shown).

	HLA-DR⁺	HLA-DR⁻	*P*
CD4⁺ p4EBP1 MFI	528 (442 – 615)	467 (401 – 529)	**<0.001**
CD8⁺ p4EBP1 MFI	546 (420 – 629)	536 (396 – 635)	0.3

Table 5.3. Maximal MTORC1 activity according to activation status.

	HLA-DR⁺	HLA-DR⁻	*P*
CD4⁺ p4EBP1 MFI (HIV negative)	528 (456 – 603)	467 (415 – 529)	**<0.001**
CD8⁺ p4EBP1 MFI (HIV negative)	567 (447 – 628)	528 (394 – 608)	**0.005**
CD4⁺ p4EBP1 MFI (HIV positive)	528 (405 – 674)	436 (297 – 518)	**0.004**
CD8⁺ p4EBP1 MFI (HIV positive)	459 (370 – 635)	548 (393 – 646)	0.1

Table 5.4. HIV negative: maximal phospho-4EBP1.

There was a good correlation between $CD4^+$ and $CD8^+$ MTORC1 activity (ρ 0.66, *P* <0.001 for phospho-4EBP1 positive proportion; ρ 0.71, *P* <0.001 for basal phospho-4EBP1; ρ 0.66, *P* <0.001 for maximal phospho-4EBP1).

5.3 Impaired MTORC1 signalling response to *in vitro* nutrient stimulation in a subset of lamina propria $CD3^+$ lymphocytes

Nutrient stimulation produced a bimodal p4E expression pattern in both $CD4^+$ and $CD8^+$ lymphocytes in many participants, with a significant proportion of cells not demonstrating any MTORC1 activity after nutrient stimulation (Figure 5.2). 7-AAD staining before and after fixation and permeabilisation in a subset of participants demonstrated that these cells were both viable up to the point of fixation, and subsequently fully permeabilised to allow nuclear staining with the phospho-4EBP1 antibody (representative experiment shown in Figure 5.3). A bimodal distribution was present in 40/70 (57.1%) of participants at baseline, and was not associated with HIV status (*P* 0.29, χ^2). The percentage of 'anorexic' lymphocytes was 20.9 (15.4 – 28.4) for $CD4^+$ cells and 17.8 (12.1 – 30.5) for $CD8^+$ cells (*P* 0.27).

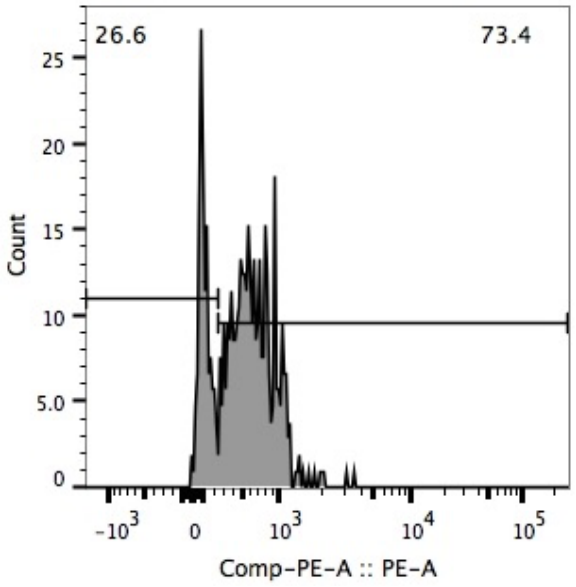

Figure 5.2. Bimodal phospho-4EBP1 pattern in lamina propria $CD4^+$ lymphocytes in response to nutrient stimulation in vitro.

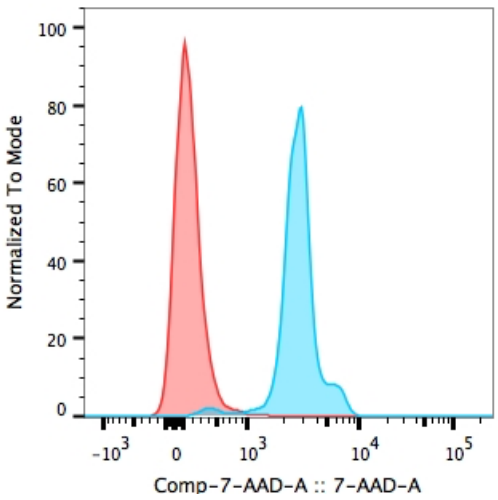

Figure 5.3. 7-AAD staining demonstrating that all LP CD4⁺ cells were viable before fixation (red histogram) and fully permeabilised after fixation & permeabilisation (blue histogram).

5.4 Effect of HIV status on lamina propria CD4⁺ T lymphocyte population

The mean number of lamina propria CD3⁺ lymphocytes harvested from biopsies in HIV negative and positive participants was similar (1778 v. 1747 cells, *P* 0.66). However, the mean number of CD3⁺ CD4⁺ cells was greatly reduced in HIV positive participants (741 v. 303, *P* 0.02). In HIV positive participants, the peripheral CD4⁺ count was strongly correlated with the proportion of lamina propria CD4⁺ lymphocytes (ρ 0.81, *P* <0.001; Figure 5.4).

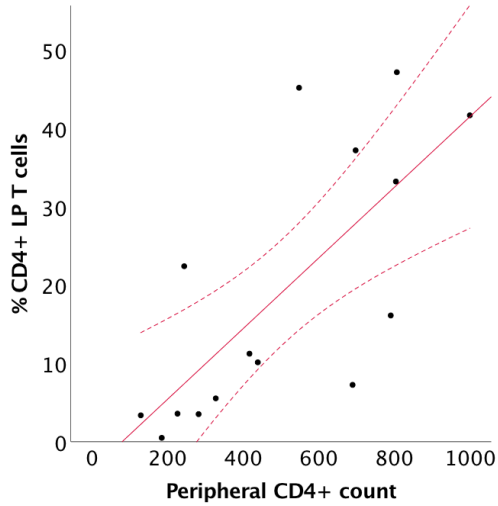

Figure 5.4. Correlation between peripheral CD4⁺ count and the proportion of CD4⁺ lamina propria T lymphocytes in HIV positive participants.

The proportion of CD4⁺ lamina propria lymphocytes was greatly reduced in HIV positive individuals (38.2 (27.9 – 54.2)% in HIV negative v. 11.6 (4.7 – 33.1)% in HIV positive; *P* <0.001; Figure 5.5); however the proportion of activated (HLA-DR⁺) lymphoctyes and the proportion of CD4⁺ T_{H1} cells was not affected by HIV status (sections 5.1 and 5.5.2).

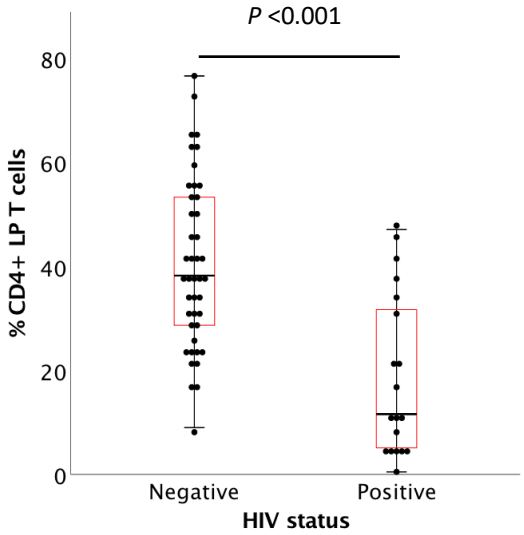

Figure 5.5. Proportion of CD4⁺ lamina propria T cells according to HIV status.

5.5 Characteristics of the lamina propria immune compartment in adults with EE

5.5.1 CD8⁺:CD4⁺ ratio

Lamina propria CD3⁺ T lymphocytes were predominantly CD8⁺ in all participants. The CD8⁺: CD4⁺ ratio at baseline was 1.6 (0.85 – 2.5) in HIV negative individuals and 7.6 (2.15 – 18.75) in HIV positive individuals (*P* <0.001). There was no correlation between CD8⁺:CD4⁺ ratio and severity of enteropathy as assessed by morphometry, CLE, or serum markers (data not shown).

5.5.2 T cell activation

The proportion of $CD4^+$ and $CD8^+$ $HLA\text{-}DR^+$ cells declined significantly over the supplementation period (Table 5.5), although no significant changes were observed when analysing by HIV status (data not shown).

	Baseline	Post	*P*
% $HLA\text{-}DR^+$ ($CD4^+$)	57.1 (42.7 – 72.4)	46.0 (35.8 – 56.2)	**0.036**
% $HLA\text{-}DR^+$ ($CD8^+$)	54.8 (42.0 – 73.0)	42.6 (30.6 – 56.9)	**0.001**

Table 5.5. Proportion of activated lymphocytes before and after supplementation.

None of the interventions resulted in significant changes in the proportion of $HLA\text{-}DR^+$ T cells ($CD4^+$: *P* 0.5 for AA v placebo, *P* 0.8 for MM v placebo; $CD8^+$: *P* 0.9 for AA v placebo; *P* 1.0 for MM v placebo).

None of the markers of MTORC1 activity (proportion of phospho-4EBP1 positive cells; basal phospho-4EBP1 MFI; maximal phospho-4EBP1 MFI) were correlated with the proportion of $CD4^+$ or $CD8^+$ $HLA\text{-}DR^+$ cells (data not shown).

5.6 Serum markers of intestinal barrier function

5.6.1 Effect of supplementation on serum markers of translocation and immune activation

A summary of baseline levels of serum CRP, sCD14 and LPS is given in Table 5.6. Levels were largely unchanged over the supplementation period in all groups. 74/74 of participants had detectable LPS at baseline, compared to 64/68 (94.1%) after supplementation. Although median levels of CRP and sCD14 were within the normal range, 22/85 (25.9%) of participants at baseline and 25/82 (30.5%) after supplementation had raised serum CRP levels. Similarly, 3/85 (3.5%) of

participants at baseline and 9/84 (10.7%) after supplementation had raised serum sCD14 levels.

	Normal	AA placebo	AA	MM placebo	MM
sCD14 / mg.ml^{-1}	<4	2.0 (1.5-2.5)	1.8 (1.4-2.4)	1.7 (1.4-2.2)	2.0 (1.5-2.6)
LPS / EU.ml^{-1}	<1	173 (88-232)	177 (104-264)	161 (79-262)	184 (107-262)
CRP / mg.ml^{-1}	<5	3.9 (1.1-9.7)	1.8 (0.7-3.4)	2.0 (0.9-8.5)	2.0 (0.7-7.9)

Table 5.6. Serum levels of sCD14, LPS and CRP at baseline. EU, endotoxin units.

None of the interventions had any effect on these markers (Table 5.7). HIV status did not influence the response to supplementation (data not shown).

CRP was weakly correlated with sCD14 at baseline (ρ 0.26, *P* = 0.017) and in all intervention groups post-intervention (ρ 0.31, *P* 0.004 across intervention groups) but neither were correlated with LPS.

Change in	AA	Placebo	*P*	MM	Placebo	*P*
LPS (EU/ml)	-36 (-136-86)	-24 (-91-58)	0.64	-55 (-165-15)	5 (-91-86)	0.11
CRP (mg/l)	0.05 (-0.8-1.7)	-0.3 (-2.9-2.0)	0.26	0.06 (-0.8-2.5)	-0.2 (-1.5-0.8)	0.19
sCD14 (mg/l)	0.3 (-0.07-0.9)	0.1 (-0.3-0.7)	0.28	0.1 (-0.3-0.7)	0.4 (-0.2-0.8)	0.37

Table 5.7. Change in LPS, sCD14 and CRP levels over time and effect of supplementation.

5.6.2 Correlations between serum markers and morphometry & endomicroscopic leak

There was a modest correlation between serum LPS levels and VH (ρ 0.28, *P* 0.038) and VA per unit mucosal length (ρ 0.31, *P* 0.022) at baseline only (Figure 5.6). There was no correlation between the serum markers and *in vivo* mucosal leak as assessed by CLE (v. LPS: ρ 0.07, *P* 0.5 ; v. sCD14: ρ –0.05, *P* 0.7; v. CRP: ρ –0.14, *P* 0.2).

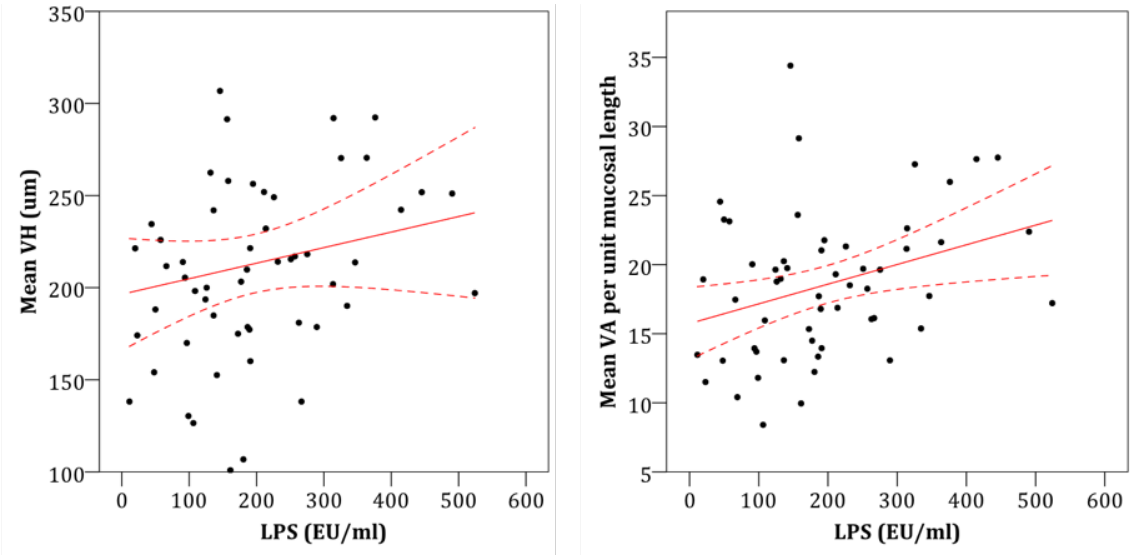

Figure 5.6. Correlation between serum LPS and morphometry at baseline.

5.6.3 Effect of HIV status on translocation and activation markers

LPS and CRP levels at baseline were unaffected by HIV status. sCD14 levels were significantly higher in HIV seropositive participants at both timepoints (Table 5.8 and Figure 5.7). Neither HIV positive nor HIV negative participants responded to any of the interventions (data not shown).

	HIV negative	**HIV positive**	*P*
sCD14 / ng.ml^{-1}	1.7 (1.4-2.0)	2.5 (2.1-3.1)	**<0.001**
LPS / EU.ml^{-1}	175 (97-262)	161 (91-266)	0.9
CRP / mg.ml^{-1}	1.9 (0.6-8.4)	4.1 (1.2-7.7)	0.1

Table 5.8. Serum levels of sCD14, LPS and CRP according to HIV status at baseline. EU, endotoxin units.

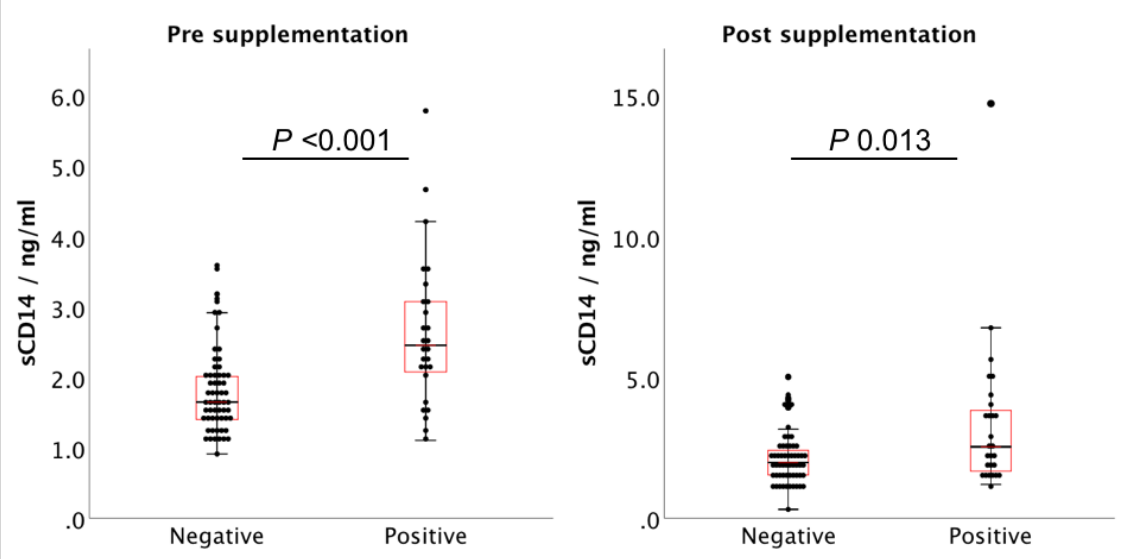

Figure 5.7. Serum levels of sCD14 according to HIV status.

5.7 Metabonomics

Metabonomic analysis was performed by Dr Jonathan Swann and Dr Jordi Mayneris, Imperial College London.

Significant OPLS-DA models were obtained for the three active groups (MM with 'AA' placebo; AA with 'MM' placebo; and both) post-supplementation compared to placebo, and also for differences over time within each treatment group. Significantly differentially excreted urinary metabolites are shown in Table 5.9 and Table 5.10, with OPLS-DA loadings plots in Figure 5.8 (p.169).

Changes by group after supplementation				
	Increased with intervention	**Reduced with intervention**	***Q²Y***	***P* for model**
MM v placebo	Panthothenate, HMB, PAGn, 4CS	NMNA, 3HPHPA	0.35	0.01
AA v placebo	BAIBA, PAGn, 4CS, citrate	No significant changes	0.35	0.01
MM+AA v placebo	Pantothenate, succinate, HMB, PAGn, fumarate, 2PY	No significant changes	0.36	0.01

Table 5.9. Significant metabonomic changes in three active treatment groups compared to placebo. 2PY = N-methyl-2-pyridone-5-carboxamide; 3HPHPA = 3-(3-hydroxyphenyl)-3-hydroxy-propionic acid; 4CS = 4-cresyl sulphate; BAIBA = β-amino-isobutyric acid; HMB = β-hydroxy-β-methylbutyrate; NMNA = N-methylnicotinic acid; PAGn = phenylacetylglutamine

Changes within group over time				
	Increased over time	**Reduced over time**	Q^2Y	***P* for model**
MM pre v post	Pantothenate, HMB, succinate, 2PY, NMND	BAIBA, 4HH, 3IS	0.54	0.01
AA pre v post	Citrate, myo-inositol, formate	Taurine, glycolate, *trans*-aconitate	0.55	0.01
MM+AA pre v post	Pantothenate, HMB, succinate, 2PY, PAGn, citrate, NMND, unknown	BAIBA, 2HIB, choline, acetylcholine, taurine, hippurate	0.79	0.01
Placebo pre v post	No significant changes	No significant changes	N/A	N/A

Table 5.10. Significant metabonomic changes in treatment groups over time. 2HIB = 2-hydroxyisobutyrate; 2PY = N-methyl-2-pyridone-5-carboxamide; 3IS = 3-indoxyl sulphate; 4HH = 4-hydroxyhippurate; BAIBA = β-amino-isobutyric acid; HMB = β-hydroxy-β-methylbutyrate; NMND = N-methylnicotinamide; PAGn = phenylacetylglutamine

To explore drivers of our primary endpoints, OPLS models were constructed to identify metabolic features associated with post-treatment villus height and post-treatment MTORC1 activation (as measured by the proportion of phospho-4EBP1 positive $CD4^+$ T cells). Significantly differentially excreted urinary metabolites are shown in Table 5.11, with a representative OPLS-DA loadings plot (for the MTORC1 model) in Figure 5.9 (p.170). Putative roles of these metabolites are given in Table 5.12 (p.172).

Outcome	**Positively correlated**	**Negatively correlated**	***P* for model**
VH	HMB, DMG, creatine, creatinine, GAA, taurine, 2PY, formate	No significant changes	0.01
MTORC1	HMB, 2HIB, 2PY, pseudouridine	4CS, Hippurate, PAGn	0.01

Table 5.11. Differentially excreted urinary metabolites correlated with post-treatment villus height and post-treatment MTORC1 responsiveness. 2HIB = 2-hydroxyisobutyrate; 2PY = N-methyl-2-pyridone-5-carboxamide; 4CS = 4-cresyl sulphate; DMG = dimethylglycine; GAA = guanidinoacetic acid; HMB = β-hydroxy-β-methylbutyrate; PAGn = phenylacetylglutamine

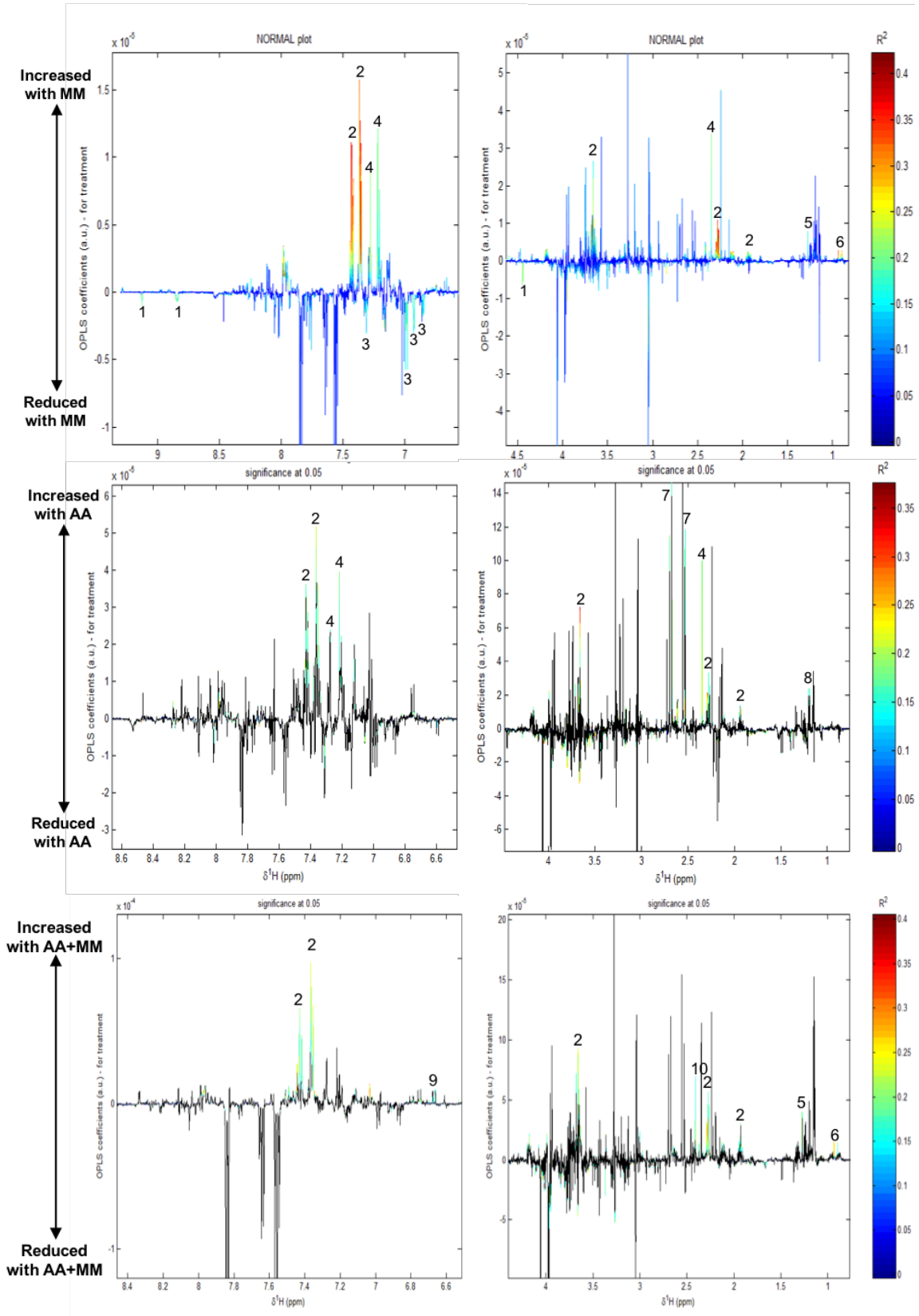

Figure 5.8. OPLS-DA loadings plots showing metabolites associated with supplementation versus corresponding placebo. Top, MM v. placebo. Middle, AA v. placebo. Bottom, AA+MM v. placebo. Metabolites with significant correlations are highlighted. 1, NMNA; 2, PAGn; 3, 3HPHPA;4, 4CS; 5, HMB; 6, Pantothenate; 7, Citrate; 8, BAIBA; 9 2PY; 10 Succinate. For abbreviations and further information see Table 5.12 .

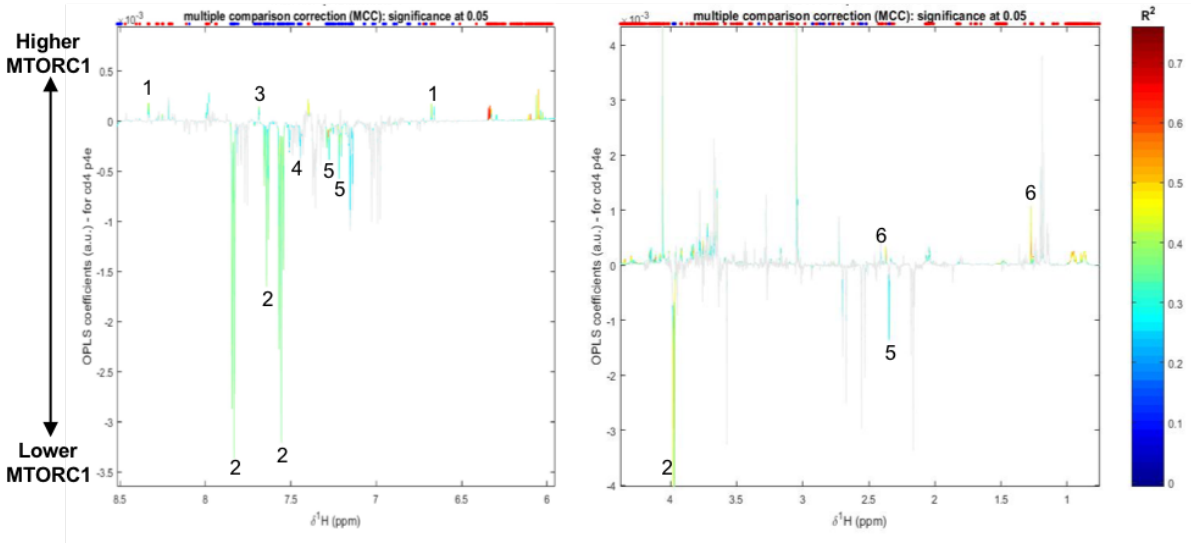

Figure 5.9. OPLS-DA loadings plots showing metabolites associated with MTORC1 activity. Metabolites with significant correlations are highlighted. 1, 2PY; 2, Hippurate; 3, Pseudouridine; 4, PAGn; 5, 4CS; 6, HMB. For abbreviations and further information see Table 5.12.

Metabolite		Pathway	Origin	Possible roles in enteropathy, stunting or nutrition	References
β-hydroxy-β-methylbutyrate	HMB	Leu	Host	Minor physiological leucine metabolite. Some evidence for promoting lean muscle mass. Positively correlated with VH in childhood EE. MTORC1 agonist.	(Borack and Volpi, 2016; Farràs et al., 2018)
Phenylacetyl-glutamine	PAGn	Phe	Co-metabolite	Produced by hepatic conjugation of L-Gln with microbially derived phenylacetic acid. Correlated with VH in childhood EE.	(Aronov et al., 2011; Farràs et al., 2018)
***N*-methylnicotinic acid**	NMNA	B3 / Trp / IDO	Host	L-Trp / Niacin (B3) metabolite. Reduced levels observed in rat model of malnutrition. Correlated with childhood wasting and underweight.	(Mayneris-Perxachs et al., 2016; Wu et al., 2010)
3-(3-hydroxy-phenyl)-3-hydroxy-propionic acid	HPHPA	Phe	Co-metabolite	Microbially derived L-Phe metabolite – particularly *Clostridia*. Possibly implicated in neuropsychiatric disorders.	(Shaw, 2010; Xiong et al., 2016)
β-amino-isobutyric acid	BAIBA	Val	Host	Lower levels associated with enhanced catch-up growth in childhood undernutrition. Anti-inflammatory and protective against oxidative stress and insulin resistance. Thermogenic.	(Mayneris-Perxachs et al., 2016)
***N*-methyl-2-pyridone-5-carboxamide**	2PY	Trp	Host	Correlated with childhood stunting.	(Mayneris-Perxachs et al., 2016)
***N*-methyl-nicotinamide**	NMND	B3 / Trp / IDO	Host	L-Trp / Niacin (B3) derivative. Hgher levels associated with enhanced catch-up growth in childhood undernutrition.	(Mayneris-Perxachs et al., 2016)
2-hydroxy-isobutyrate	2HIB		Co-metabolite	Correlated with childhood stunting and VH in childhood EE.	(Farràs et al., 2018; Mayneris-Perxachs et al., 2016)
Pantothenate		B5	Host	Required for lipid biosynthesis. Correlated with childhood stunting.	(Mayneris-Perxachs et al., 2016)

Taurine		Cys	Co-metabolite	Low levels associated with childhood stunting.	(Semba et al., 2016)
4-cresyl sulphate	4CS	Tyr	Co-metabolite	Correlated with childhood stunting.	(Mayneris-Perxachs et al., 2016)
Citrate		TCA	Host	Negatively correlated with childhood stunting.	(Mayneris-Perxachs et al., 2016)
Succinate		TCA	Host	Correlated with VH in childhood EE.	(Farràs et al., 2018)
Fumarate		TCA	Host	Unknown	
Myo-inositol		PL	Host	Enhances insulin sensitivity. Precursor to membrane phospholipids.	
Formate			Co-metabolite	Byproduct of acetate metabolism.	
Hippurate			Co-metabolite	Correlated with childhood wasting and underweight.	(Mayneris-Perxachs et al., 2016)
4-hydroxyhippurate			Co-metabolite	Correlated with VH in childhood EE.	(Farràs et al., 2018)
Glycolate				Unknown	
***Trans*-aconitate**		TCA		Unknown	
3-indoxyl sulphate	3IS	Trp	Co-metabolite	Correlated with childhood stunting and VH in childhood EE.	(Farràs et al., 2018; Mayneris-Perxachs et al., 2016)
Choline				Required for lipid membrane stability and lipid metabolism.	
Acetylcholine		TCA		Unknown	
Guanidinoacetic acid	GAA			Creatine precursor; livestock muscle enhancer supplement	(Ostojic, 2015)

Table 5.12. Significant metabolites identified through metabonomic analysis: putative biological roles. B3, niacin; B5, pantothenate; IDO, indolamine 2,3-dioxygenase; TCA, tricyclic acid cycle; PL, phospholipid synthesis.

5.8 Enterohumoral trophic signalling assessed by plasma GLP2 levels

Serum GLP2 levels were similar across all treatment groups, and did not change significantly over the supplementation period. GLP2 levels were not altered by any of the interventions (Table 5.13). GLP2 levels were suppressed in HIV positive individuals (Table 5.14 and Figure 5.10), although HIV status had no effect on response to supplementation (data not shown).

	AA	Placebo	*P*	MM	Placebo	*P*
Baseline GLP2 (ng/mL)*	0.76 (0.0 – 1.4)	0.92 (0.49 – 1.4)	0.44	0.75 (0.0 – 1.5)	0.88 (0.51 – 1.4)	0.86
Δ GLP2 (ng/mL)	0.21 (-0.57 – 0.63)	0.34 (0.0 – 0.52)	0.44	0.25 (-0.27 – 0.57)	0.25 (-0.05 – 0.59)	0.66

Table 5.13. Effect of AA or MM supplementation on fasting serum GLP2 concentrations. *Normal range not established.

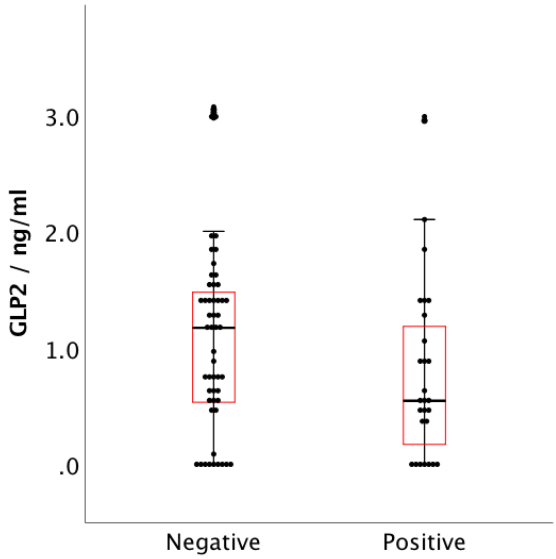

Figure 5.10. Serum GLP2 concentrations according to HIV status.

	HIV negative	HIV positive	*P*
GLP2 / ng.ml^{-1}	1.2 (0.5 – 1.5)	0.6 (0.0 – 1.3)	**0.047**

Table 5.14. Baseline GLP2 levels by HIV status.

Furthermore there was a moderate negative correlation between morphometry and GLP2 levels in HIV positive individuals only. Although small intestinal absorptive area as measured by morphometry was not correlated with GLP2 levels in the study population as a whole, there was a moderate negative correlation with both VH (ρ -0.53, *P* 0.02) and CD (ρ -0.46, *P* 0.046) in HIV positive individuals at baseline.

5.9 Global nutritional status and body composition

5.9.1 Effect of interventions on body composition

Baseline anthropometric and nutritional markers are given in Table 5.15. None of the interventions influenced any of these nutritional outcomes (Table 5.16).

	AA	**Placebo**	**MM**	**Placebo**
MUAC	27.3 (25.3 – 30.2)	28.5 (25.8 – 32.0)	28.3 (25.4 – 31.3)	27.9 (25.4 – 31.3)
Grip strength	31.8 (24.7 – 39.2)	29.2 (27.1 – 36.9)	30.7 (27.5 – 36.9)	31.2 (25.7 – 37.9)
Body fat %	24.5 (17.2 – 34.4)	30.9 (19.3 – 40.6)	30.3 (17.6 – 38.1)	28.5 (17.3 – 36.1)
BMI	22.5 (19.7 – 25.0)	22.4 (20.5 – 27.6)	23.0 (20.0 – 26.4)	21.5 (19.8 – 25.8)

Table 5.15. Baseline anthropometric characteristics by intervention group.

Change in:	**AA**	**Placebo**	***P***	**MM**	**Placebo**	***P***
MUAC	0.3 (-0.8 – 1.2)	0.2 (-1.0 – 1.4)	0.86	0.1 (-0.8 – 1.4)	0.3 (-1.0 – 1.0)	0.79
Grip strength	0.0 (-2.8 – 1.6)	-0.9 (-3.8 – 1.1)	0.25	-0.9 (-3.8 – 1.3)	-0.6 (-2.4 – 0.9)	0.64
Body fat %	-1.2 (-3.4 – 0.7)	-0.8 (-3.7 – 1.3)	0.69	-1.1 (-3.6 – 1.5)	-0.9 (-3.5 – 0.6)	0.89
BMI	-0.4 (-0.7 – 0.4)	0.0 (-0.7 – 0.3)	0.95	-0.4 (-0.8 – 0.2)	-0.0 (-0.5 – 0.5)	0.13

Table 5.16. Effect of intervention on body composition.

5.9.2 Effect of HIV

Despite similar lean mass percentage and other anthropometric markers, HIV positive individuals had lower grip strength at baseline only (Table 5.17).

	HIV negative	HIV positive	*P*
MUAC	28.0 (25.7 – 31.0)	27.9 (25.1 – 32.0)	0.90
Grip strength	34.4 (27.0 – 39.1)	28.7 (25.7 – 33.0)	**0.04**
Body fat %	27.7 (16.9 – 36.2)	31.7 (21.6 – 38.6)	0.31
BMI	22.6 (20.0 – 26.0)	21.4 (19.8 – 26.0)	0.51

Table 5.17. *Baseline nutritional characteristics by HIV status.*

Body fat percentage moderately correlated with CRP in HIV negative participants only (ρ 0.46, *P* <0.001; Figure 5.11), but not other markers of enteropathy or leak including morphometry and endomicroscopy (data not shown).

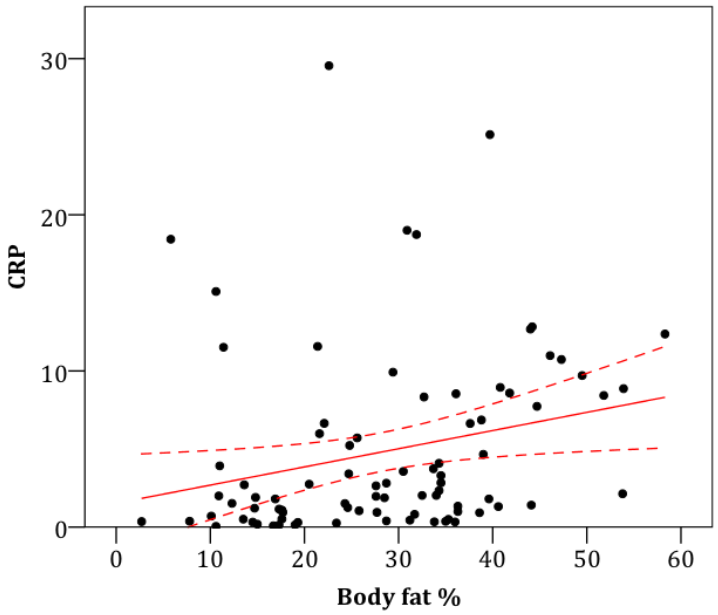

Figure 5.11. Correlation between serum CRP and body fat percentage at baseline in HIV negative participants.

CHAPTER 6

DISCUSSION

6.1 Overview

Environmental enteropathy is a sub-clinical alteration in small intestinal mucosal architecture and function, widely prevalent in the tropics and in disadvantaged communities. A randomised, double blind, placebo-controlled trial was completed in a particularly disadvantaged sub-Saharan urban community with little food security or access to sanitation, clean water or health facilities. The trial explored several factors thought to influence enteropathy: nutrition and the mucosal immune response to nutrients; gut permeability; systemic and mucosal immune inflammation; dysbiosis; and trophic enterohumeral signalling.

This was a complex interventional phase II trial undertaken in a low income country. The trial was successfully completed as planned according to the trial protocol. This is the largest clinical study of confocal laser endomicroscopy ever undertaken, with *in vivo* CLE examinations completed in 84 participants at two timepoints. It is also the first time that the mucosal immune compartment in EE has been studied by flow cytometry, and the first study of nutrient and MTORC1 signalling in human intestinal immune cells.

Participants were representative of the adult population from which they were drawn, and were predominantly young and female. Importantly, the proportion of HIV positive participants was similar to the population prevalence (30%). Although the study was conducted in healthy adults, the participants had clear evidence of stunting and of significant asymptomatic small bowel enteropathy.

In addition to lifestyle markers of deprivation (lack of access to chlorinated / boiled water; low levels of education; low levels of household hygiene and sanitation), the high prevalence of non-pathogenic intestinal parasites (as well as helminths and *Giardia* in excluded participants) reflects the unsanitary conditions in this community.

Despite widespread undernutrition in this population, a significant proportion of the study population were overweight or obese (occasionally morbidly so) despite harbouring significant enteropathy. This likely reflects one sequela of the 'impoverished gut' hypothesis (Guerrant et al., 2013), where impaired gut barrier function results in a chronic systemic pro-inflammatory response and the metabolic syndrome. Women were disproportionately overweight, which is opposite to the pattern observed in Western populations (although the overall prevalence of overweight is significantly lower in Zambia). In the UK, for example, male and female levels of overweight are 68% and 58% respectively (Health and Social Care Information Centre, 2016). This has concerning implications at a population health level, given that both stunting and the metabolic syndrome are inheritable phenomena, and the increased perinatal and maternal morbidity and mortality associated with maternal overweight.

The supplements were well tolerated and compliance appeared to be high. This was assessed using supplement counts every four weeks; although directly observed therapy is the gold standard for confirming compliance, this was not feasible in an interventional study of this size and nature. Some of the significant metabolites in the AA and MM groups (pantothenate in MM supplemented; PAGn – a glutamine co-metabolite – in AA supplemented) may be indirect evidence of good compliance.

6.2 Summary of findings

Although there was evidence for an effect of the interventions on villus morphology and barrier dysfunction as hypothesised, the supplements did not modify mucosal lymphocyte MTORC1 nutrient sensing.

The novel findings arising from this study include:

- Amino acid supplementation compared to placebo increased villous height and villous cross-sectional area, but this change was not correlated with changes in serological or endomicroscopic signs of intestinal barrier dysfunction.

- The combination of amino acids and micronutrients, taken together but not separately, did appear to reduce gut permeability assessed by confocal endomicroscopy.
- Although mucosal T lymphocyte MTORC1 activity was not influenced by either intervention, post-supplementation lamina propria $CD4^+$ MTORC1 activity was correlated with villous height and cross-sectional area.
- Serological measures of immune activation and microbial translocation (CRP; sCD14; LPS) did not change with any of the interventions. LPS was correlated with VH at baseline only.
- Although MTORC1 activity was not altered by supplementation, $CD4^+$ T cell nutrient responsiveness was correlated with TBET expression in $CD4^+$ T cells (T_{H1} phenotype). There was a high proportion of LP T_{H1} cells.
- Levels of GLP2, a trophic enterohumoral peptide, were generally paradoxically depressed and were not influenced by supplementation, including with amino acids.
- The lamina propria $CD8^+$:$CD4^+$ ratio was reversed, with a $CD8^+$ dominant population compared to normal.
- There appears to be a seasonal effect on MTORC1 responsiveness in LP lymphocytes, although there was no correlation with morphometry which also exhibited a seasonal variation.
- A number of metabolites were identified which correlated with VH and MTORC1 activity; of these, the tryptophan metabolite 2PY and the leucine metabolite HMB were also affected by supplementation (although not by amino acid supplementation alone).
- In contrast to effects on morphometry, some of these results were affected by HIV status.
- No effects were observed on body composition assessed using a number of methods.

Taken together, these findings provide further insight into the immunological, microbiological and functional abnormalities characterising EE. Although AA supplementation improved VH and VA, and supplementation with both AA and MM improved endomicroscopic barrier dysfunction, there were few correlations between the histological severity of enteropathy or endomicroscopic severity of

barrier dysfunction and any other markers of enteropathy. There was an intriguing correlation between villus height (and small intestinal histology) and $CD4^+$ lymphocyte nutrient responsiveness.

6.3 Enteropathy characteristics in the study population

6.3.1 Severity of enteropathy at baseline

Morphometry, confocal endomicroscopy, and serological markers were consistent with a moderate to severe enteropathy, despite no evidence of loss of epithelial integrity under white light endoscopy. Morphometry confirmed marked villus atrophy and crypt hypertrophy. The villus-to-crypt ratio was in several cases <1, and was normal (≥ 3) in none. Severe and almost universal intestinal dysfunction was observed by confocal endomicroscopy. Pathological barrier dysfunction has been noted in other small bowel diseases including quiescent Crohn's disease, coeliac disease, and irritable bowel syndrome (Fritscher-Ravens et al., 2014; Kiesslich et al., 2012; Leong et al., 2008; Neumann et al., 2012), but the gross abnormalities observed here are remarkable by their severity and universality. For example, quiescent Crohn's is characterised by pathological endomicroscopic findings in up to 30% of fields in individual patients, in comparison to the great majority of involved fields in this study.

6.3.2 Microbial translocation and immune activation

Serum markers of bacterial translocation such as LPS and immune activation such as sCD14 and CRP have been used indirectly to assess the level of translocation *in vitro*. These markers were elevated in a significant proportion of healthy asymptomatic individuals in this study, but were unaffected by the study interventions. LPS was almost universally detectable; over a quarter of participants had elevated levels of CRP; and up to 10% of participants had elevated levels of sCD14. Interestingly, sCD14 levels were significantly higher in HIV

positive participants despite other markers of enteropathy being similar, suggesting that other subtle abnormalities are at work in HIV infection.

The importance of low grade chronic inflammation has been implicated in the metabolic syndrome. In stunted infants, elevated inflammatory markers are correlated with serum markers of intestinal damage and with suppressed growth factor secretion, providing a mechanism which links immune activation due to intestinal damage to impaired growth in children (Prendergast et al., 2014).

The relevance of these markers in adults with enteropathy is not fully understood, but in western populations has been implicated in the metabolic syndrome. The 'impoverished gut' hypothesis extrapolates this to impoverished communities, where chronic immune activation in combination with early life epigenetic imprinting predisposes these individuals to obesity and metabolic disorders including diabetes which is increasingly observed in these populations despite the lack of classical risk factors for these conditions (Guerrant et al., 2013).

6.3.3 The lamina propria immune compartment

6.3.3.1 $CD8^+$ predominance in the $CD3^+$ compartment

The investigation of the mucosal immune compartment in EE by flow cytometry has not previously reported. The lamina propria was heavily skewed towards $CD8^+$ ($CD3^+$ $CD4^-$) T cells, with $CD8^+$ cells outnumbering $CD4^+$ cells 1.6:1 in HIV negative individuals and 7.6:1 in HIV positive individuals, in contrast to normal lamina propria where $CD4^+$ predominate. In the only previous study to assess the lamina propria in EE (in Gambian children), LP T lymphocyte density was assessed using immunohistochemistry (Campbell et al., 2003a). $CD3^+$ cells were dramatically increased compared to UK controls, and the numbers of $CD4^+$ and $CD8^+$ cells were estimated to be similar. Although the ratio is skewed further in this current study, both studies strikingly demonstrate the reverse of 'healthy' lamina propria, where $CD4^+$ cells predominate. It is interesting to speculate that the proportion of $CD8^+$ cells may increase over time in appropriately exposed environments. This may reflect the excessive T cell mediated cytotoxic tissue response resulting in the disordered mucosal architecture observed in EE; however, there was no

correlation between $CD8^+$:$CD4^+$ ratio and morphometric, endomicroscopic, or serological measures of enteropathy.

It is interesting to contrast these studies in individuals with EE with patients with coeliac disease, a histologically similar enteropathy, where 80% of lamina propria T cells are $CD4^+$ (Jöhrens et al., 2010). Both are characterised by lamina propria T cell infiltration and a cytotoxic T cell inflammatory response. It is likely that both LP $CD4^+$ and $CD8^+$ lymphocytes are increased in EE (with proportionately greater $CD8^+$ cells, explaining the inverse ratio observed), whereas in coeliac disease the increased infiltrate is due predominantly to $CD4^+$ recruitment. The lamina propria $CD8^+$ dominance has not been described in other conditions, and the drivers and consequences of this require further study.

6.3.3.2 Activated lymphocytes

In this study, approximately half of LP $CD4^+$ and $CD8^+$ T lymphocytes were activated (HLA-DR^+). There are little data on LP lymphocyte activation in the proximal small bowel, and no recent studies using flow cytometry. However, a study investigating the proportion of duodenal / proximal jejunal lamina propria $CD4^+$ HLA-DR^+ lymphocytes by immunohistochemistry found only 33% and 8% of $CD4^+$ and $CD8^+$ lymphocytes (respectively) were activated in adults with untreated coeliac disease, compared to 20% and 12.5% in controls (Griffiths et al., 1988). On the other hand, a previous study of adults with EE in this population, where HLA-DR status was also assessed immunohistochemically, found that 87% of LP $CD3^+$ cells were HLA-DR^+ (Veitch et al., 2001). Interestingly the proportion of $CD3^+$ HLA-DR^+ cells observed in relatively affluent South Africans in the same study is comparable to the that seen in the current study. It is possible that improved living standards over the last fifteen years has resulted in a reduction in immunological stimulation. However, EE is convincingly characterised by an activated lamina propria T cell immune response in addition to a highly activated epithelial immune compartment; and this is markedly more pronounced than the lamina propria activation observed in coeliac disease, a histologically similar enteropathy.

Mucosal lymphocytes are traditionally seen as controlling and influencing the mucosa. It is interesting to note that the severity of enteropathy (as assessed

histologically) was not correlated with lamina propria T_{H1} or $CD8^+$ activation (as assessed by HLA-DR positivity), and similarly that changes in morphology were not correlated to HLA-DR positivity. It would be interesting to compare and contrast these findings with coeliac disease; to my knowledge this has not been investigated previously.

6.3.3.3 T_{H1} predominance in the $CD4^+$ compartment

The LP $CD4^+$ population was predominantly of a T_{H1} phenotype, as observed in normal lamina propria and in coeliac disease (Jöhrens et al., 2010). This is in keeping with previous observations that EE is characterised by T_{H1}-mediated T cell cytotoxicity (Campbell et al., 2003a; Veitch et al., 2001). Counterintuitively, however, these T_{H1} cells had a predominantly resting phenotype ($HLA\text{-}DR^-$). Possible explanations for this include immunological senescence following chronic over-stimulation, or suppression in a tolerogenic environment. Given that overall $CD4^+$ activation was approximately 50%, by definition non-T_{H1} $CD4^+$ cells will be highly activated and it is interesting to speculate that this may reflect a highly activated T_{reg} population.

6.3.3.4 'Anorexic' lymphocytes

A majority of participants had lamina propria T cells which exhibited a bimodal response to nutrient stimulation. This could most easily be explained by non-viable cells (which would not respond to nutrient stimulation) or by failure of the permeabilisation step (so that the target phospho-4EBP1 antigen was not accessible for staining). However both of these possibilities were excluded with the use of a viability dye, which confirmed that these cells were viable before fixation and adequately permeabilised before staining. This subset of lymphocytes could not be characterised further; they may represent senile, anergic or pre-apoptotic cells, which are metabolically inactive. This phenomenon was not observed in peripheral T cells from healthy UK donors, and further work is required to determine if this phenomenon is specific to environmental enteropathy, lamina propria T cells, or both. There was no correlation to any other

flow cytometric findings such as T cell activation, the proportion of T_{H1} cells, or $CD8^+$:$CD4^+$ ratio.

6.3.4 Seasonal variability

Seasonal variation in mucosal structure and / or intestinal permeability and microbial translocation in this population has previously been demonstrated (Kelly et al., 2004). A reduction in small bowel absorptive area over the rainy season (which coincided with the supplementation period) was again observed. This has anecdotally been attributed to increased pathogen load resulting in increased mucosal inflammation, secondary to deteriorating water and sanitation in the rainy season. In this study no functional changes in gut function were observed – LPS, sCD14, CRP and endomicroscopic leak were unchanged over the supplementation period, somewhat arguing against this. However, a reduction in dietary quality and quantity during this time (colloquially known as the 'hungry season') may also be relevant, particularly with regards to nutrient sensing pathways.

6.4 Impact of supplementation on the intestinal barrier in EE

6.4.1 Effects of amino acid supplementation

AA supplementation prevented a seasonal deterioration in villous height and cross-sectional area. Crucially, the observed effect on mucosal histology was not associated with in any changes in gut barrier function. Microbial translocation (measuring LPS) and systemic immune activation (measuring serum CRP and sCD14) were all unaffected by the interventions, and were not correlated with the histological severity of enteropathy. An interaction effect was observed on barrier permeability as assessed by CLE: barrier leak reduced in participants who received both active supplements. The reduction in permeability observed does not appear to be functionally relevant, as there was no correlation with other markers of

barrier dysfunction such as LPS, sCD14, and CRP; this suggests that greater improvements in permeability than those observed are required to reduce microbial translocation.

These findings can be explained by hypothesising that different pathophysiological mechanisms are driving villus atrophy and intestinal permeability. Villus height in the proximal small bowel (determined by enterocyte replication, migration and apoptosis) might be rate-limited by MTORC1 signalling and the enteral availability of nutrients such as glutamine, leucine and tryptophan. Conversely, pathological permeability may be predominantly driven by dysbiosis and inflammation. If improvements in histology and barrier function are limited to the proximal small bowel (the major site of nutrient absorption), then residual gut dysfunction present in the distal small bowel and colon may be enough to negate any benefits in the proximal bowel. This hypothesis will be difficult to test until less invasive, but still reliable, methods of evaluating the whole small intestinal mucosa are available.

6.4.2 Effects of multiple micronutrient supplementation

In the current study, no effects of MM supplementation on the villus unit were identified. This is in contrast to a *post hoc* analysis of a trial of vaccine immunogenicity in healthy adults with EE in this population, which suggested that high dose multiple micronutrient supplementation improved villus height (Louis-Auguste et al., 2014). The findings from this study are likely to be more reliable as this morphometry was a primary outcome in this study, as opposed to a secondary retrospective analysis. Furthermore, our previous study used histological samples taken at a single timepoint during the Zambian dry season, whereas the current study looked for changes on a background of a seasonal deterioration in morphometry. It is possible that the severity of the enteropathy in the rainy season would have negated any beneficial effects of the micronutrient supplement.

6.4.3 Relationship between barrier dysfunction and morphometry

Little is known about the correlation between small intestinal histology and intestinal permeability, as the great majority of work has been conducted in children with non-invasive tests. In this study, permeability in healthy but stunted adults was measured in two complementary ways: directly by endomicroscopy and indirectly by levels of serum markers. A positive correlation between serum LPS and small intestinal absorptive area at baseline (reflected in VH and VA per unit mucosal length) was observed, without any correlations with other serum and confocal markers of barrier dysfunction (although numbers were relatively small). This suggests that, at least up to a certain extent, a reduced surface area may in fact be protective against microbial translocation: individuals with blunted villi have less area for translocation to occur, resulting in lower levels of microbial translocation. A relatively mild reduction in absorptive capacity is sacrificed in order to reduce the degree of systemic immune activation and chronic inflammation.

The lack of other correlations may be explained by hypothesising that the physiological improvements from a relatively unaffected proximal small bowel may be completely negated by a dysfunctional distal bowel, as discussed above. Furthermore, subtle changes in permeability would not necessarily be detectable by endomicroscopy, as high levels of fluorescein leak would still be observed.

In a previous study in this population, the histological severity of enteropathy was inversely correlated with intestinal permeability as assessed by urinary lactulose recovery (Kelly et al., 2004). In a recent study we identified a weak correlation between serum LPS and endomicroscopic barrier defects / leak, as well as a U-shaped correlation between serum LPS and morphometry (Kelly et al., 2016). The positive correlation between VH and serum LPS seen here would be consistent with the upstroke of this correlation.

Although sCD14 and CRP levels were correlated with each other, neither were correlated with levels of LPS. Of these markers, LPS is direct measure of bacterial translocation, whereas sCD14 and CRP are host-related factors; it is possible that immune dysregulation / tachyphylaxis in individuals with EE means that levels of

these inflammatory markers / mediators are paradoxically suppressed and may not be correlated with translocation in a linear manner.

Over all, the correlation between VH and serum LPS needs to be taken with caution as the strength of the correlation was modest (ρ 0.31), no effect was observed post-supplementation (although any correlation may have been obscured by the positive effect of AA supplementation on VH), and there was no association with the other markers used. However, this is some further evidence for a 'protective' role of mucosal atrophy in EE, and represents a conceptual shift in the pathogenesis of EE.

6.5 Potential role of MTORC1 signalling

The role of MTORC1 signalling in lymphocytes of the mucosal immune compartment has not previously been investigated. These first results in humans support a role of MTORC1 signalling promoting villus growth. Amino acid but not micronutrient supplementation improved VH compared to placebo, without influencing mucosal T lymphocyte MTORC1 activity. However the correlation between post-supplementation VH and $CD4^+$ MTORC1 activity in participants receiving amino acids suggests that immune cell MTORC1 signalling may indirectly influence the epithelial compartment. Interestingly, a similar positive correlation was also observed in post-supplementation VH and $CD4^+$ MTORC1 activity in the MM supplement group. This may suggest that amino acid or micronutrient supplementation may prime the villus compartment for growth, in response to permissive nutrient-replete signals from the immune compartment.

The trophic effect of AA supplementation on the intestinal epithelium may be mediated trough enterocyte / intestinal stem cell MTORC1 signalling. For example, Glutamine is a well known enterotrophic amino acid but its mechanism of action is unknown. Intriguingly, however, a number of *in vitro* and animal models have suggested that MTORC1 inhibition (by dietary or pharmacological means) results in mucosal atrophy (Dias et al., 1998; Nakamura et al., 2012; Yi et al., 2015). Furthermore, animal and *in vitro* models suggest a role for intestinal epithelial MTORC1 signalling in protecting against apoptosis and LPS-induced cell death.

These findings are of obvious relevance for EE, and may explain the protective effect on VH observed in AA supplementation.

Preliminary work for this study attempted to investigate epithelial MTORC1 signalling using immunohistochemistry. In surgically resected small intestinal tissue, phospho-rS6 (an alternative marker of MTORC1 activity) was diffusely and indiscriminately expressed in the epithelial barrier, in contrast to previous work in mice where activity is predominantly limited to the crypts (Nakamura et al., 2012; Yilmaz et al., 2012). We hope to conduct further work to examine the role of MTORC1 signalling in the villous unit in future work.

6.5.1 Lamina propria lymphocyte MTORC1 signalling and T_{H1} differentiation

This is thought to be the first investigation of MTORC1 signalling in intestinal lymphocytes. MTORC1 signalling has critical roles in innate and adaptive immune cell (particularly $CD4^+$ T lymphocyte) differentiation and response. In peripheral $CD4^+$ lymphocytes, MTORC1 activity promotes a T_{H1} (effector) phenotype, whereas MTORC1 inhibition results in a T_{reg} (anti-inflammatory) phenotype (Powell et al., 2012). T cell activation also requires MTORC1 activity which can only occur in a nutrient-rich environment.

It was postulated that amino acid supplementation would augment lymphocyte MTORC1 signalling and therefore promote TBET expression / T_{H1} differentiation. However amino acid supplementation had no effect had MTORC1 signalling or TBET expression. The amino acid dosages are likely to have ensured adequate delivery to the proximal small bowel. It is possible that the lack of response observed is a phenomenon secondary to the chronic immune stimulation in EE (e.g. anergy or senescence). Alternatively, isolated nutrient stimulation may be an insufficient stimulus for MTORC1 activation in this immune cell population in individuals with EE.

On the other hand, the proportion of nutrient-responsive LP lymphocytes correlated with the proportion of $CD4^+$ $TBET^+$ T cells, consistent with a role for MTORC1 signalling in effector cell differentiation. TBET is the canonical T_{H1}

transcription factor and is necessary and required for T_{H1} differentiation and function (i.e. IFNγ production). The mucosal immune compartment is predominantly inhabited by experienced (differentiated) immune cells; however these cells exhibit plasticity under appropriate stimuli and immune signalling conditions. Therefore the population of TBET$^+$ cells observed may include non-classical 'mixed' immune cells expressing TBET, in addition to classical T_{H1} cells. In other systems, these cells have T_{H1}-like effector functions including IFNγ production. Further work is required to establish the precise immunophenotype, function and relevance of these TBET$^+$ cells, but this work suggests that MTORC1 signalling promotes a T_{H1} or T_{H1}-like phenotype in mucosal lymphocytes.

6.5.2 MTORC1 signalling by T cell subtype

Basal MTORC1 activity was higher in CD8$^+$ compared to CD4$^+$ cells in HIV negative participants only. Although the proportion of nutrient-responsive lymphocytes was not affected by activation status, activated lymphocytes (HLA-DR$^+$) had higher maximal phospho-4EBP1 levels compared to resting (HLA-DR$^-$) cells. This implies that activation augments MTORC1 synergistically with nutrient stimulation. The higher MTORC1 activity observed would promote a transcriptional and metabolic program to support effector functions. Due to experimental design, basal phospho-4EBP1 levels according to activation status and phospho-4EBP1 levels in TBET$^+$ cells could not be measured.

Although the proportion of responsive cells was similar at both study timepoints, basal and maximal MTORC1 levels (assessed by p4E MFI) declined significantly over the supplementation period. Interestingly the proportion of activated CD4$^+$ and CD8$^+$ cells also declined over the supplementation period. The biological significance of this finding is unclear.

Due to LP CD4$^+$ depletion in HIV positive participants, there were insufficient CD4$^+$ cells for analysis in many cases and the sample size was small. Conclusions regarding MTORC1 activity cannot be confidently drawn from this group of individuals; however, AA supplementation appeared to enhance CD4$^+$ MTORC1

responsiveness in HIV positive individuals. These findings need to be replicated in the first instance.

6.6 Effect of HIV on the mucosal immune compartment

Lamina propria $CD4^+$ depletion is an early and durable consequence of HIV infection. In this current study population, as has previously been observed, overall LP T cell numbers were similar but the proportion and number of $CD4^+$ cells was dramatically reduced in HIV positive individuals, even when peripheral $CD4^+$ counts were robust.

Studies in Western cohorts suggest that early HAART initiation as the most important determinant of mucosal $CD4^+$ reconstitution (Allers et al., 2016; Guadalupe et al., 2003); furthermore peripheral reconstitution in these studies does not predict mucosal reconstitution. Zambian treatment guidelines at the time of the study advised initiating treatment for all asymptomatic adults once peripheral $CD4^+$ count was ≤500 (Zambia Ministry of Health, 2014), based on WHO guidelines (World Health Organization, 2013). Most HIV positive participants in the study were established on HAART. Although information of $CD4^+$ count and WHO HIV stage at diagnosis / initiation of treatment was not available, it is likely that many patients were diagnosed later than would be the case in Western populations. It is therefore surprising that the proportion of mucosal $CD4^+$ cells was strongly correlated with the peripheral $CD4^+$ count. It therefore appears that the predictors (and therefore pathophysiology) of mucosal $CD4^+$ T cell reconstitution in this population is different; it is possible that the additional mucosal and systemic immune stimulation resulting from EE may be contributing. The correlation between peripheral and mucosal $CD4^+$ counts has not previously been described in a population with a high prevalence of EE and may have important implications in treatment of HIV in impoverished communities.

HIV viral load was not measured as this was not an aim of the study. Although the HIV virus may have a directly cytopathic effect on the intestinal epithelium (Epple et al., 2010), its effects on the mucosal immune system is likely to be predominantly due to its role in manipulating the $CD4^+$ compartment.

6.6.1 Effect of HIV on intestinal barrier structure and function

Importantly, despite its effect on the mucosal immune system, HIV status did not affect any of the outcomes measured, and neither peripheral nor mucosal $CD4^+$ counts correlated with any of the outcomes. However, there were some subtle differences at baseline between HIV positive and negative participants.

No histological or endomicroscopic differences between HIV positive and negative participants were observed. The lack of effect of HIV on histology and on barrier permeability may seem surprising. However, the data presented here are entirely consistent with earlier work (Kelly et al., 2004) which showed that only in advanced AIDS did villus height fall below the range consistent with environmental enteropathy, whereas crypt depth was increased earlier in HIV infection. It is likely that in industrialised countries HIV may impact on villus morphology, but that in LMICs any such subtle effect is undetectable alongside the environmental enteropathy which is virtually ubiquitous. Although the number of HIV positive participants was small, this suggests that HIV infection (when well controlled as defined by peripheral $CD4^+$ count, and irrespective of viral load) is not a primary determinant of enteropathy severity in HIV positive individuals, despite its effects on the mucosal immune compartment.

Serum sCD14 concentrations, but not other markers of translocation and immune activation, were significantly higher in HIV positive individuals compared to HIV negative ones. sCD14 is released by activated monocytes, for example in response to LPS. The elevated levels observed here may reflect a mucosal barrier dysfunction which is subtly more pronounced in HIV positive individuals, and / or subtle over-activation of systemic innate immune signalling. Of note, elevated sCD14 levels are correlated with excess morbidity and mortality in HIV positive patients in Western populations (Hunt et al., 2014; Sandler et al., 2011). The excessive mortality of some HIV patients with EE initiating HAART in impoverished populations may be partially explained by a dysregulated innate immune response; sCD14 may provide a useful biomarker for risk stratification.

6.7 Insights into host-microbial interactions

Metabonomic analysis revealed a number of insights which may merit further investigation. A number of host- and microbiota-derived metabolites were significantly correlated with VH and/or $CD4^+$ T lymphocyte MTORC1 activity. Metabonomics has only recently been applied to EE / undernutrition and this is the first study to investigate the metabonomic profile of adults. There has been one previous study investigating the urinary metabonome with small intestinal histology in severely malnourished and stunted Zambian children (Farràs et al., 2018), one non-invasive study investigating correlations with stunting and growth in Brazilian children (Mayneris-Perxachs et al., 2016), and one non-invasive study investigating responses before and after treatment for severe acute malnutrition in Malawian infants (Giovanni et al., 2016). Intriguingly, many of the significantly correlated metabolites observed in the current study have previously been identified as markers of enteropathy and / or gut dysfunction and / or stunting in one or more of these studies.

6.7.1 Markers of muscle mass and metabolism

β-hydroxy-β-methylbutyrate (HMB) is of particular interest. HMB was positively correlated with both MTORC1 activity and VH. HMB is a physiological leucine metabolite, with some studies supporting a role for HMB in enhancing lean muscle mass and strength (Borack and Volpi, 2016). Intriguingly, it is a potent MTORC1 agonist. However, despite the presence of leucine in the supplement, HMB levels were not augmented by amino acid supplementation alone, although it was paradoxically increased by MM (with and without AA) supplementation. These results corroborate a recent study in Zambian children, where a positive correlation between HMB and VH was also noted (Farràs et al., 2018).

The correlation between creatinine and creatine and VH is intriguing, as both are markers of lean body mass, and may be more sensitive markers of lean body mass than global body composition analysis. The positive correlation may suggest a correlation between epithelial health and a shift towards anabolism in a reduced inflammatory state. Of note, a positive correlation between creatinine and VH was

also observed in a Zambian paediatric population (Farràs et al., 2018), and creatinine was negatively correlated with stunting in a Brazilian paediatric population (Mayneris-Perxachs et al., 2016). Similarly, guanidinoacetic acid (GAA) is a creatine precursor, and was positively correlated with VH.

6.7.2 Tryptophan and its metabolites

Physiologically and microbially derived metabolites of tryptophan include *N*-methylnicotinamide (NMND), *N*-methylnicotinic acid (NMNA), 3-indoxyl sulphate (3IS), and *N*-methyl-2-pyridone-5-carboxamide (2PY). Tryptophan-derived metabolites were correlated with MM supplementation (with or without AA supplementation), but not with AA supplementation alone. These included 2PY and NMND which increased with supplementation, and NMNA which reduced with supplementation. 3IS was reduced in the MM + placebo group only. It therefore appears that micronutrient supplementation modifies host and /or microbial tryptophan pathways, and that the amount of tryptophan in the amino acid supplement was insufficient to alter host / microbial metabolic pathways.

In this study, 2PY was correlated with both VH and MTORC1 activity. In the Brazilian study, 2PY was correlated with stunting, but was also a predictor of good catch-up growth. Although the histological severity of enteropathy was unknown in the Brazilian study, good catch-up growth must reflect low systemic inflammation and a pro-anabolic environment, which would be consistent with a lesser degree of villus atrophy. The association with MTORC1 activity may reflect MTORC1 activation by tryptophan-related metabolites.

Although no correlations with VH were observed, NMND levels increased and NMNA levels reduced with intervention. This may have subtle beneficial effects, as in the Brazilian study NMND was positively correlated with catch-up growth, whereas NMNA was negatively correlated.

The tryptophan / indoleamine 2,3-dioxygenase (IDO) / kyneurine immunometabolic axis is currently a subject of great interest. Tryptophan is partly metabolised through host IDO 1 and 2 to produce kynurenines, and this pathway may be subverted by microbial tryptophan uptake and metabolism (McGaha et al.,

2012; Murray, 2016). High levels of IDO activity promote regulatory immune responses and T_{reg} differentiation and inhibit T_{eff} differentiation, mediated through kynurenine and related tryptophan metabolite production (Yan et al., 2010), activation of GCN2 via tryptophan depletion (Munn et al., 2005), and inhibition of MTORC1 (Metz et al., 2012).

In stunted children with EE, plasma tryptophan levels are correlated with growth (Semba et al., 2016), and the kynurenine to tryptophan ratio (KTR, with high ratios indicative of high IDO activity) is correlated with systemic markers of inflammation and inversely correlated to growth (i.e. high KTR and presumed greater IDO activity associated with greater stunting) (Kosek et al., 2016), suggesting that microbial manipulation of tryptophan signalling and inhibition of kynurenine production may be relevant in the pathophysiology of EE. The differences observed in this study also suggest a possible role for tryptophan signalling in modulating the villus compartment and immune cell nutrient sensing.

6.7.3 Other metabolites

Dimethylglycine (DMG) is a marker of choline flux. In this study it was positively correlated with VH. This is consistent with the Brazilian study where it was negatively correlated with stunting (Mayneris-Perxachs et al., 2016).

A number of other metabolites correlated with VH, MTORC1 activity, or the interventions were identified. These can be broadly classed as amino acid-derived (e.g. taurine; 4-cresyl sulphate; phenylacetylglutamine), TCA cycle intermediaries (e.g. succinate; formate), or microbial co-products (e.g. pseudouridine; hippurate). Although some of these have been identified in undernourished / stunted populations previously, the significance of these findings in this study is unclear.

6.8 Enterohumoral signalling in EE: role of GLP2

GLP2 is physiologically secreted in L cells in the distal small bowel in response to luminal amino acid delivery. GLP2 stimulates villus growth which increases small

intestinal absorptive area and thus nutrient (including amino acid) absorption. AA supplementation however did not augment GLP2 levels. This could be because, despite the presence of enteropathy and reduced absorptive area, all of the amino acid load would have been absorbed before reaching the site of amino acid sensing and GLP2 secretion in the distal ileum.

Fasting GLP2 levels measured here were an order of magnitude lower than published values obtained from normal volunteers (Hartmann et al., 2000; Russo et al., 2012). Although some of this variability may be explained by different kits, the lowest results observed in normal adults (5ng/ml) was obtained using the same ELISA kit as used in the current study (Russo et al., 2012). These values are still several times larger than the median results observed in this study. GLP2 levels are also paradoxically suppressed in children with severe acute malnutrition with severely reduced small bowel absorptive areas (Amadi et al., 2017) and have also been noted to be low in adults with EE in this population (Kelly et al., 2016), providing strong evidence that the GLP2 axis is dysregulated in EE. The suppressed levels suggest that there is a failure of normal sensing / secreting mechanisms rather that end-organ resistance. However, the lack of trophic signalling may have a protective role, by reducing the amount of permeable mucosa available for microbial translocation.

GLP2 levels were significantly lower in HIV positive individuals compared to HIV negative individuals. Villus height and crypt depth were also inversely correlated to serum GLP2 levels in HIV positive participants only. As the severity of enteropathy (as assessed by morphometry) was similar in HIV positive and negative participants, it is unlikely that this reflects a difference in absorptive area; rather this may reflect a difference in how nutrient signals to the distal small bowel are regulated in HIV.

6.9 Anthropometry

Baseline anthropometrics in this community encapsulate the burgeoning concern of the 'impoverished gut' – despite the relative food insecurity in this impoverished population, there is a high prevalence of overweight and obesity. It is postulated

that the abnormal microbial translocation and systemic immune activation observed in EE results in a disordered (pro-inflammatory) metabolic phenotype, comparable to the dysregulation observed in the metabolic syndrome. Of all the measures of intestinal barrier dysfunction used in this study, only CRP correlated with body composition, and then only in HIV negative participants. CRP is a non-specific marker of immune activation from whatever cause, and this correlation may therefore reflect the degree of global immune activation observed in EE, as a sum of the sub-clinical contributions of a number of pro-inflammatory pathways (reflected, for example, in LPS or sCD14).

The AA supplement contained the leucine and tryptophan equivalent of 150g of beef (US Department of Agriculture, 2018), and is likely to have provided a significant increase in daily essential amino acid intake. The lack of response on lean body mass may reflect pathological diversion of nutritional resources in EE in a catabolic state.

6.10 Study strengths

This study was conducted in the context of a double blind, randomised, placebo-controlled trial, and the differences observed between treatment groups can therefore be attributed solely to those treatments. Compliance was good and there were no problems with the administration of the study which would limit its reliability or cast doubt on its conclusions.

To my knowledge, this is the largest clinical study of confocal laser endomicroscopy ever undertaken, with *in vivo* CLE examinations completed in 84 participants at two timepoints.

One of the strengths of this study was the histological samples that were obtained for analysis. This study obtained a number of tissue types from well characterised healthy adults, including small intestinal biopsies. The vast majority of studies investigating EE rely on indirect / non-invasive measures of barrier dysfunction, most usually sugar absorption / permeability studies. This study builds on our

previous studies which have investigated the links between the histological and functional abnormalities observed in EE.

Interventional studies in LMICs should ideally investigate interventions which, if shown to be useful, can be relatively easily scaled up to phase III trials or population-level introduction within the logistical and resource limited environment. This trial investigated the effects of supplementation in a representative sample taken from a community which mirrors the problems of poverty and poor sanitation present throughout sub-Saharan Africa and beyond in populations with EE. The interventions themselves were simple, safe, well tolerated, easily administered and cheap.

6.11 Study weaknesses

6.11.1 Carbohydrate and micronutrient absorption assays

The standard assay of epithelial barrier function in studies of EE is the sugar absorption / permeability assay. In particular, xylose absorption is most relevant to the assessment of global small intestinal absorptive function. Although imperfect, it remains in use because it is safe and non-invasive. However, analysis is not widely available and there are technical limitations. This study aimed to explore the use of novel and alternative methods of assessing the small intestinal barrier, in particular using CLE as in *in vivo* method. In order to assess the validity of CLE in the study of EE, and in particular to assess absorptive function, a solution containing D-xylose (absorbable), L-rhamnose (non-absorbable), lactulose (non-absorbable), and inulin (non-absorbable) was administered at the time of endoscopy, and baseline and interval urine samples were obtained and stored at –80°C. Furthermore, plasma samples to assay zinc and selenium uptake following instillation of a second test solution were also obtained. Unfortunately due to insurmountable technical and servicing problems with the optical emission spectrophotometer in Zambia (for the zinc and selenium uptake assays) and HPLC in the UK (for the urinary sugar recovery assays) it was not possible to complete these assays in the timescale of this work. Although assessment of zinc and selenium levels in the context of this trial would be interesting, the sugar

permeability / absorption studies are important to place the results of intervention in context with other studies of enteropathy, and with the utility of CLE in this condition. It is hoped that these samples will be analysed and results will become available in 2019 to enable a more complete picture of the effects of the interventions.

6.11.2 Sample size and power

The morphometry assessments used established techniques and protocols, and sample size was calculated using preliminary data from a previous study (Louis-Auguste et al., 2014). This assumed a power to detect 15% difference in morphometry with the interventions when n=28 in each group (active treatment v. placebo). The sample size required to detect differences in CLE and MTORC1 signalling was unknown, and the required sample size was assumed to be similar to the morphometry. In effect it would have been extremely difficult to increase the trial size much further given the constraints of the invasive and resource-intensive nature of interventional trials involving advanced endoscopy. Sample size was adversely affected by inadequately orientated histological sections and by low rates of viable mucosal lymphocyte recovery to the point of flow cytometric acquisition due to the destructive effect of methanol permeabilisation and relative paucity of tissue. The interventions did not result in any changes in any of the secondary outcomes measured, although the study was not powered for these outcomes.

6.11.3 Poor discriminatory ability of CLE

CLE was a promising technique to examine epithelial barrier defects in real time, and has shown reproducibility and prognostic validity in conditions including IBD and IBS. This study for the first time used CLE to look for differences in barrier function following an intervention. The data from this data show that functional mucosal barrier defects are almost universal; significant fluorescein leak was seen in the majority of duodenal fields assessed in most participants. This is in stark contrast to previous work by others in other inflammatory conditions; in clinically

and endoscopically quiescent IBD, for example, small intestinal endomicroscopic leak was observed in up to 30% of fields and in only 5% of normal controls (Kiesslich et al., 2012). The almost universal presence of high levels fluorescein leak encountered in individuals with EE means that the CLE scoring system used had a low ability to discriminate between individuals or over time.

6.11.4 Lack of assessment of epithelial MTORC1 activity

The mechanisms by which nutrition influences the intestinal mucosa is largely unknown. This study investigated the role of nutrient sensing by MTORC1 in mucosal T lymphocytes. No effect of supplementation was observed in the T lymphocytes but was seen in the epithelial compartment (assessed by morphometry), and this was correlated with T lymphocyte MTORC1 responsiveness. However epithelial MTORC1 signalling could not be assessed due to the difficulties with developing an assay that was sufficiently sensitive and discriminatory to detect subtle differences in MTORC1 activity within the intestinal epithelium. This could probably only be done with IHC, as epithelial cells are not amenable to flow cytometric analysis. However, in preparatory work, staining with the available MTORC1 pathway antibody (directed against phospho-rS6) was present diffusely throughout the epithelium and therefore could not provide a semi-quantitative assessment of epithelial MTORC1 activity. Furthermore, satisfactory co-staining with lymphocyte markers was not possible with several antigen retrieval protocols. This technique was therefore not pursued further.

6.12 Methodological comments

6.12.1 Morphometry reproducibility

Unfortunately, in contrast to previous studies in this population it was not possible to obtain well orientated histology sections for several participants, reducing the sample size available for morphometric analysis. However, the baseline

morphometric measurements of the small intestinal epithelial compartment in this study and in a previous study in the same population (Louis-Auguste et al., 2014) are strikingly similar, suggesting that the morphometry methodology is valid.

6.12.2 CLE as a method to assess gut permeability

In this study CLE was used as an *in vivo* method to assess intestinal barrier dysfunction. The barrier dysfunction noted on CLE was significantly greater than that observed in other classical enteropathies (Kiesslich et al., 2012; Leong et al., 2008; Neumann et al., 2012). The scoring system used here was easy to use and had excellent inter-observer agreement. However, the gross fluorescein leak observed may have obscured more subtle abnormalities which might provide better discriminatory ability. For the first time in adults, CLE provided direct, *in vivo* evidence of the bizarre and highly abnormal mucosa which until now has only been observed histologically, but it was not possible to quantify this reproducibly.

6.12.3 Assessment of MTORC1 activity

MTORC1 activity in lamina propria T cells was measured by quantifying levels of phospho-4EBP1 by flow cytometry. Three measures of MTORC1 activity were measured: basal activity (phospho-4EBP1 MFI after a period of nutrient deprivation *in vitro*); maximal activity (phospho-4EBP1 MFI after a period of nutrient stimulation *in vitro*); and the proportion of nutrient-responsive cells (the proportion of phospho-4EBP1 positive cells following a period of nutrient stimulation *in vitro*, compared to the unstimulated / nutrient deprived control). All three of these measures are potentially biologically relevant; for example T cells with high basal levels of MTORC1 activity may be primed to perform effector functions when stimulated, and cells with high maximal levels of MTORC1 activation in response to nutrient stimulation may provide a greater amplitude response to stimulation. The proportion of cells responding to nutrient stimulation likely reflects the population of cells that are available to divide / differentiate / respond to an immune stimulus.

In vitro, LP immune cells exhibited a clear response to nutrient stimulation. However no change in response was seen following *in vivo* supplementation. The doses chosen ensured high delivery to the proximal small bowel, and the delivery of amino acids and micronutrients to the lamina propria immune compartment is likely to have been sufficient. This suggests that chronic stimulation of the lamina propria T cell MTORC1 system with exogenous (dietary) nutrients does not upregulate MTORC1 signalling.

6.12.4 Identification of T lymphocyte subsets

A number of methodological considerations meant that not all T cell subsets could be positively identified.

- $CD3^+$ $CD4^-$ lymphocytes were assumed to represent $CD3^+$ $CD8^+$ lymphocytes. This is likely to be valid as $CD3^+$ $CD4^-$ $CD8^-$ cells (which would have an identical appearance in these experiments) are a rare population in the lamina propria.
- $CD3^+$ $CD4^+$ $TBET^+$ cells were assumed to be T_{H1} cells, although $CD4^+$ lymphocytes with mixed functions (e.g. T_{H1} – Th17 hybrids) would appear identical. However, studies of lymphocyte plasticity suggest that hybrid cells expressing TBET exhibit T_{H1}-like properties; similarly, TBET positive cells were assumed to be functional (i.e. effector cytokine production such as IFNγ in response to appropriate immunological stimuli.
- HLA-DR alone was used as an activation marker.
- No functional assays were performed.

6.12.5 Methods for assessing intestinal permeability

It was intended to measure intestinal permeability by urinary lactulose recovery. Due to technical reasons it has not yet been possible to undertake this, although appropriate samples were taken and stored during the study. The results of this assay will allow us to determine which method of assessing gut permeability is most relevant, but all three permeability assays (endomicroscopy; serum

biomarkers; urinary sugar recovery) may have pathophysiological relevance and are likely to give complementary information.

Abnormal sugar permeability is correlated both with bacterial translocation and growth faltering in children (Campbell et al., 2003b); however this has not been demonstrated in adults (although there is no obvious reason to suspect this does not occur). Mechanistically, sugar uptake studies measure the paracellular uptake of normally non-absorbable simple carbohydrate molecules; the relevance of this to the passage of much larger and more complex molecules such as LPS has not been demonstrated and may not be directly linked.

On the other hand, CLE has been studied exclusively in adults, and pathological fluorescein leak into the gut lumen has been shown to imply inward leakage of large molecules (at least up to MW 10,000kDa) (Kiesslich et al., 2012). Furthermore, in a prospective study of quiescent, endoscopically normal terminal ileal Crohn's disease (another condition characterised by endoscopically normal mucosa), the presence of endomicroscopic leak was clinically relevant and predicted risk of clinical overt relapse (Kiesslich et al., 2012).

6.13 Conclusions and future directions

In this double-blinded placebo-controlled randomised controlled trial, supplementation with AA but not MM increased villus height and villus area compared to placebo on a background of a seasonal deterioration in small intestinal architecture. AA in combination with MM also reduced the pathological intestinal barrier permeability observed *in vivo* by CLE. The pronounced correlation between post-treatment phospho-4EBP1 expression in lamina propria $CD4^+$ cells and villus height suggests that nutrient sensing by the MTORC1 pathway in the mucosal immune compartment plays a part in determining villus morphology, but we could not detect evidence that this determines the response to amino acid supplementation.

Baseline and post-supplementation assessments demonstrate that the enteropathy in this population is significant, and is characterised by a number of

immunological, histological, metabonomic, and enterohumoral abnormalities. The study has suggested novel mechanisms in which nutrient status can influence the mucosal immune compartment, for example by influencing TBET expression / T_{H1} differentiation. Furthermore, for the first time in humans, it has provided insights into nutrient sensing in lamina propria T lymphocytes, by demonstrating differential responsiveness to nutrient stimuli depending on activation status and T cell type, for example. Despite previous *in vitro* evidence of a role for nutrient supplementation in determining T cell fate, *in vivo* supplementation with amino acids did not alter T cell fate or MTORC1 signalling.

Metabonomic profiling identified potentially physiologically relevant metabolites that were altered by the interventions studied, and metabolites that were associated with intestinal epithelial morphology and MTORC1 nutrient sensing. One of these in particular, HMB, was upregulated by the supplements and was positively correlated with villus height and MTORC1 signalling, and has previously been identified as being of particular interest for its role in promoting lean muscle mass. Changes were identified in numerous host-microbial metabolic pathways, many of which have previously been identified as being implicated in EE. Future work will need to identify the pathophysiological relevance of these perturbations. Furthermore, although this study summarises some of the functional consequences of the presumed dysbiosis in EE, it will be important to complete the assessment of the microbiota with other "omic" methodologies using stool specimens taken in the course of the study.

Although there was no correlation between serum and *in vivo* endomicroscopic measures of barrier dysfunction, both methodologies are potentially relevant and require further investigation to determine method is most relevant for quantifying gut barrier dysfunction. The methods are conceptually different and are likely to provide complementary information about the abnormalities observed. Assessments of micronutrient (zinc and selenium) absorption and macronutrient (carbohydrate) absorptive capacity will hopefully complete the assessment of gut barrier function in the coming months.

The AMAZE study has shown that a combination of selected amino acid and micronutrient supplementation can result in some histological and functional changes in adults with EE. This is believed to be the first time that a therapy for EE

has been demonstrated to have positive effects on its histological features in the context of a randomised controlled trial. It has recently been proposed that the assessment of environmental enteropathy should be considered in several domains – villus morphology, permeability, microbial translocation, mucosal inflammation, systemic inflammation, malabsorption, and dysbiosis (Keusch et al., 2014; Owino et al., 2016). These data suggest that therapies for EE may have an impact on one or two of these domains (in this case, villus atrophy and *in vivo* barrier permeability) without necessarily affecting others; furthermore, it is unknown which of these pathophysiological domain(s) is / are most relevant to growth in children, and to controlling systemic inflammation and reducing intergenerational deficits in adults. Future work involving long-term follow up will need to address this.

Due to the superadded problems of under-resourced healthcare systems and healthcare infrastructure in the developing world, successful therapeutic interventions for EE must be both pathophysiologically effective and simple to implement. The interventions used in the AMAZE study were safe, well tolerated, and cheap, and may therefore be suitable for further study as a clinical intervention. This work has also provided further insights into the complexity of the relationship between nutrition, mucosal immune function, and intestinal health. For as long as this relationship remains poorly understood, it will continue to present fascinating opportunities for further study.

APPENDICES

1. Patient information sheet (English version) 205
2. Consent form 209
3. Case reporting forms 210
    - Enrolment
    - Endoscopy
    - Anthropometry
    - Monthly review
    - *Ad hoc* / unplanned review
4. ImageJ morphometry macro 219
5. Incubation media for MTORC1 flow cytometry assay 221

Amino acids and / or multiple Micronutrients in Adult Zambians with environmental Enteropathy: effects on intestinal structure & function and host-microbiome interactions. A randomised placebo controlled trial (AMAZE)

We would like to invite you to be part of this research project. You should only agree to take part if you want to, it is entirely up to you. If you choose not to take part there won't be any disadvantages for you and you will hear no more about it. Choosing not to take part will not affect your access to treatment or services in any way, now or in the future.

Please read or listen to the following information carefully before you decide to take part; this will tell you why the research is being done and what you will be asked to do if you take part. Please ask if there is anything that is not clear or if you would like more information.

If you decide to take part you will be asked to sign a consent form to say that you agree. You are still free to withdraw at any time and without giving a reason.

Why we are doing this research

Almost all children and adults who live in Misisi, like millions of other people in Africa and around the world who do not have easy access to clean water or toilets, often have problems with bowel inflammation. Although this inflammation doesn't cause any symptoms or make you feel unwell, we now think that it causes significant problems with growth and development.

Over the last twenty years, we have done several research projects into this problem in Misisi, and we have learned a lot about this condition. Unfortunately, there is still a lot that is unknown, and there are still no treatments for the inflammation.

Why we are doing this new research study

One of our previous studies suggested that giving people some extra vitamins every day may help with some of the signs of the inflammation. It is also possible that the inflammation can be reduced by giving people some simple proteins to take. We would like to investigate the effects that these simple proteins and vitamins have on bowel inflammation in more detail. If the results are promising, then future trials will see if people should be given these supplements routinely.

What the study involves

You will be asked to take two supplements every day – either a vitamin pill or inactive pill, and either a simple protein powder or energy powder. You will take the same supplements every day for four months. You will not be able to tell which pill contains the vitamins, or which powder contains the proteins. Most people (three out of every four) will receive either the vitamins or the protein powder or both, but some people (one out of every four) will get the energy powder and inactive capsule. This is so that we can be sure that any benefits are truly due to the vitamins and / or proteins, rather than chance. Whether you get vitamins, protein, energy powder or inactive pill is up to pure chance, and you will not be told which substance you are getting. Even your doctors will not know until the end of the study.

It is very important that you take the supplements every day, as this is the only way to be sure whether or not they will work. For this reason it is also very important that the supplements are only taken by you and not shared with or given to anyone else. Although the supplements are very safe in healthy adults, we do not know what their effects are in children, or in people that we have not assessed.

The supplements can be taken by themselves, or mixed into or sprinkled on top of nshima or water. It is important that you take all of the supplements every day. It is probably easiest for you to take them with your main meal, but you can take them at another time of day if you forget.

The investigations you will have

Before you enter the study, you will have a full medical assessment and examination. We will also ask for a stool sample to make sure you don't have a worm infection. If you do, we will give you treatment for this.

If we identify any medical problems, we will give you treatment for this, or refer you to a specialist at UTH.

We will bring you to UTH before you start and after you finish the supplements after four months. At UTH, we will examine the inside of your food pipe, stomach and start of your small bowel using a small flexible camera (endoscopy), and take some tiny tissue samples of the lining of your bowel at the same time. We will also take two blood samples (which is about half a mouthful), two urine samples, a saliva sample and a stool sample on the same day. Finally, we will do some specialist tests of body composition, to see how much of your body is made up of water, muscle and fat. All these tests are completely safe and painless.

These tests will take most of a day and we will give you 80K to compensate you for the time you have given to come for them.

About the endoscopy camera test

The endoscopy test feels strange but is not painful. It is a very safe test. Professor Kelly and his team are very experienced and have performed over 2500 endoscopies on people from Misisi without any major problems.

You will have the endoscopy test lying down on your side. You can keep your clothes on. We will give you some numbing spray to swallow and give you an injection to make you feel sleepy and relaxed. Many people fall asleep and can't remember the test afterwards. The flexible camera is about as thick as your finger and so can be easily swallowed, although it will feel strange. We will take some tiny tissue samples of the lining of the bowel, which you won't feel as there is no sensation in this part of the bowel. The tissue samples are about the size of a small scratch, and are so small that they don't damage your bowel at all.

Your stomach must be empty for the test to be done safely, so you can't have anything to eat or drink on the day of the endoscopy until after the procedure, but we will give you lunch afterwards.

If you are interested, we can show you the endoscopy unit and equipment at UTH so you have a better idea of what is involved.

HIV testing

Effective medicine for HIV is now freely available in Zambia, and it is much more effective if discovered and treated before it gets advanced and causes symptoms. Knowing your HIV status is important for your health and the health of your family. It is therefore a good idea to have an HIV test regardless of whether or not you want to take part in the study, and whether or not you

feel unwell. To take part in the study, you will need to have an HIV blood test, because HIV can affect bowel inflammation and how it reacts to the supplements. If you are found to have HIV, we will refer you to specialists who will investigate you further and start treatment if you need it.

The result of the HIV test is completely private and confidential. We are not allowed to tell your friends or family, or anyone else, what the result is without your permission, even if it is negative.

Who can take part in the study

Most adults who live in Misisi B section can take part. We cannot include children, women who are pregnant or breast feeding, or people who are unable to have an endoscopy test.

What we do with your samples

We only use the samples you provide for research. Any samples that we do not need for research will be destroyed. We have no need to use the samples you provide for any other purpose, including selling them or giving them to people who are not involved in the research. It is also against the law to do so.

We do many of the research analysis in the laboratories at UTH. Some of the experiments will need to be done by Professor Kelly's research team in the UK, so some of your samples may be sent there. His team in the UK will also either use the samples for research, return them to Zambia, or destroy them. Like in Zambia, his UK team is not allowed to do anything with the samples that isn't to do with research, including selling them or giving them to people who aren't involved in the research.

We do not use all the samples immediately, and we often have to store them for use in the future. Samples are normally stored in freezers which are kept securely in the laboratories, which only Professor Kelly and his team have access to. When we have completed all the experiments that we need for the study, we will see if any samples are left over. If so, we would like to keep them for use in future research studies, so that we may not need to repeat the procedures to get more samples. The same rules on what we can do with the samples, and who can use them, will apply in any future research. Before we could use your samples for research in the future, we would first need to ask permission from the Ethics Committee in the University of Zambia who will make sure that it is ethical to do so.

We do many different experiments on the samples you provide, and if you are interested we can demonstrate and explain some of these to you, including how the samples are stored.

What we do with the information you provide

During the research we collect information about you, for example how much you weigh, how old you are and how much inflammation is in your bowel. We store the information on computers and remove your name and other ways of identifying you, so that there is no way of telling who the information refers to. The only way we can find out is by looking up your research number in a book which is kept in a locked office at UTH, which only Professor Kelly has access to. The researchers who analyse your samples will not be able to tell whose samples they are analysing.

Any and all information you give us is confidential. This means that we are not allowed to tell anyone about you, even if they are friends or family. When the information we collect is analysed, it becomes impossible to tell who gave the samples, so it is not possible for you to be identified from your samples.

We think that the knowledge we gain in this study will give us a lot of important information into what causes the bowel inflammation and what can be done about it. We would like to share this information with other scientists and governments in conferences and publications. Nobody who hears or reads about the research will be able to tell that the information or samples came from you.

How this study might benefit you

While you are in the study, you and your family will have free health care at the Misisi clinic. If you have an illness that we cannot treat at the clinic, we will send you for further tests or treatment by specialists at UTH, which you will not have to pay for.

The camera endoscopy test is the best test for examining your food pipe and stomach. Sometimes people have problems like ulcers that don't cause any symptoms, but which can sometimes still be serious. If we find that you have any of these problems when we do the endoscopy camera test we will investigate and treat them.

It is important for your health and the health of your family that you know your HIV status, even if you feel completely well. Effective medicine for HIV is now freely available in Zambia, and it is much more effective if discovered and treated before it gets advanced.

Insurance and what to do if you have questions or concerns

Queen Mary University of London has agreed that if you are harmed as a result of your participation in the study, you will be compensated, provided that, on the balance of probabilities, an injury was caused as a direct result of the intervention or procedures you received during the course of the study. These special compensation arrangements apply where an injury is caused to you that would not have occurred if you were not in the trial. These arrangements do not affect your right to pursue a claim through legal action.

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form.

If you have any questions or concerns about the study or how it is conducted, you can contact Professor Kelly's team in the Misisi clinic at any point. If you are not happy to do this, or are not happy with the advice you get from Professor Kelly's team, you can contact the University of Zambia Biomedical Research Ethics Committee, University of Zambia School of Medicine Ridgeway Campus, PO box 50110.

Thank you.

Consent form (English)

Please complete this form after you have read or listened to the Information Sheet and/or listened to an explanation about the research.

Amino acids and / or multiple Micronutrients in Adult Zambians with environmental Enteropathy: effects on intestinal structure & function and host-microbiome interactions. A randomised placebo controlled trial. (AMAZE)

UNZABREC reference: 007-11-14

1. I have read or listened to the Participant Information Sheet for this study (version 2.1, dated 18th December 2014).
2. The research has been explained to me. I understand what the research involves and what is required of me.
3. I understand that I will have a blood test for HIV.
4. I understand that samples I provide may be sent abroad for research purposes only, and that after use the samples will either be returned to Zambia for storage or destroyed.
5. I consent to the long term storage of any samples I provide (including blood, urine, stool, small bowel fluid and endoscopic biopsies) in secure facilities at the University of Zambia after this research study has finished, for possible ethically approved research projects in the future.
6. I understand that there is no obligation for me to participate in the research, and that I do not have to give a reason for not participating.
7. I understand that if I decide at any time during the research that I no longer wish to participate, I can notify the researchers involved and be withdrawn from it immediately, without giving a reason.
8. I consent to the processing of my personal information for the purposes of this research study. I understand that such information will be treated as strictly confidential at all times and that any identifiable information about me will be available only to members of the study team.

Participant's Statement:

I _____ have read or listened to statements 1-8 above and I agree to take part in the study.

Signed: Date:

Witnessed: Date:

Investigator's Statement:

I _____ confirm that I have carefully explained the nature, demands and any foreseeable risks of the proposed research to the volunteer.

Signed: Date:

Name _____

House number B __ __ __

Date __ __ / __ __ / __ __ (dd/mm/yy)

Inclusion criteria (TICK IF CRITERIA MET)

- ☐ AGED BETWEEN 18 AND 60 YEARS
- ☐ ABLE TO GIVE INFORMED CONSENT
- ☐ RESIDENT IN MISISI STUDY AREA

Exclusion criteria (TICK IF CRITERIA MET THEN **DO NOT RECRUIT**)

- ☐ NOT WILLING TO UNDERGO HIV TEST
- ☐ WARFARIN OR BLEEDING DISORDER
- ☐ UNABLE TO HAVE ENDOSCOPY SAFELY
- ☐ PREGNANT OR BREAST FEEDING
- ☐ BMI < 18
- ☐ UNWILLING TO CONSENT FOR LONG TERM STORAGE OF SAMPLES

Temporary exclusion criteria (POSTPONE FURTHER INVESTIGATIONS UNTIL CRITERIA NO LONGER APPLY)

- ☐ DIARRHOEA IN THE LAST 4 WEEKS (>3 WATERY STOOLS FOR >24 HOURS)
- ☐ USED ANTIBIOTICS OR NSAIDs WITHIN LAST 4 WEEKS (TAKEN REGULARLY FOR >72 HOURS)
- ☐ HELMINTH / WORM INFECTION

Date eligible to start study (if not today) __ __ / __ __ / __ __

4 WEEKS AFTER LAST LOOSE STOOL

4 WEEKS AFTER LAST ANTIBIOTIC OR NSAID USE

6 MONTHS AFTER FINISHED ANTI-HELMINTHIC COURSE

Demographics and social history

Age ___ ___ years Sex Male / Female

Occupation _____ Time in Misisi ___ ___ years

Marital status Single / Married / Widowed / Divorced / Separated / Co-habiting

Smoking Never / Ex / Rarely / Weekly / Daily

Year started ___ ___ ___ ___ / Year stopped ___ ___ ___ ___

Alcohol Never / Monthly or less / 2-3 times a month / Weekly / 2-3 times a week / Daily

Alco drink Beer / Kachasu / Other _____

Level of education None / Primary / Secondary / College / University

Household characteristics

Does someone in your household own the house you live in? No / Yes

Do you have electricity? No / Yes

Do you have a radio? No / Yes

Do you have a cell phone? No / Yes

Do you have any pets which live indoors? No / Yes

How often do you…

Boil water Never / Cholera / Rains / Most days / Always

Add chlorine Never / Cholera / Rains / Most days / Always

Household hygiene score

Cleanliness 0 / 1 / 2

Water storage 0 / 1 / 2

Food storage 0 / 1 / 2

Hand washing 0 / 1 / 2

Sanitation 0 / 1 / 2 **HHS total** ___ ___ **/ 10**

Please complete food frequency questionnaire for last month (see separate sheet)

Past medical history		**Year diagnosed** (yyyy)
HIV status	Negative / Positive / Unknown	__ __ __ __
TB	No / Pulmonary / Abdominal / Meningitis / Other _____	__ __ __ __
Hepatitis / jaundice	No / Yes	__ __ __ __
Stroke	No / Yes	__ __ __ __
Surgery	No / Yes _____	__ __ __ __
Diabetes	No / Diet controlled / Tablet / Insulin / Both	__ __ __ __
Heart disease	No / MI / Heart failure / Other _____	__ __ __ __
Hypertension	No / Yes	__ __ __ __
Asthma	No / Yes – no admissions / Yes – previous admissions	__ __ __ __

Other major illness 1	_____	__ __ __ __
Other major illness 2	_____	__ __ __ __
Other major illness 3	_____	__ __ __ __

Current symptoms

Current symptom 1 _____	Duration 1	(days) __ __ / (weeks) __ __
Current symptom 2 _____	Duration 2	(days) __ __ / (weeks) __ __
Current symptom 3 _____	Duration 3	(days) __ __ / (weeks) __ __

Current medications			**Year started** (yyyy)
Drug 1 _____	Dose 1_____	prn / od / bd / tds / qds	__ __ __ __
Drug 2 _____	Dose 2_____	prn / od / bd / tds / qds	__ __ __ __
Drug 3 _____	Dose 3_____	prn / od / bd / tds / qds	__ __ __ __

Antihypertensives	No	/	Yes				__ __ __ __
Heart failure	No	/	Yes				__ __ __ __
Diabetes	No	/	Tablets	/	Insulin	/ Both	__ __ __ __
ART	No	/	Yes				__ __ __ __
Aspirin	No	/	Yes				__ __ __ __
Asthma	No	/	Yes				__ __ __ __
Other _____			Indication _____				__ __ __ __

Do you take any unprescribed / self-prescribed / over-the-counter / traditional medicines?

No / Yes _____

Observations

Height __ __ __ **cm** **HR** __ __ __

Weight __ __ . __ **kg** **BP** __ __ __ / __ __ __

MUAC __ __ . __ **cm** **RR** __ __

Karnofsky ___ ___ ___ **/ 100** **Grip strength** __ __ . __ **kg** **LEFT / RIGHT**
(TEST **NON-DOMINANT** SIDE)

Physical examination (CIRCLE ALL THAT APPLY)

Breathlessness None / After exertion / At rest

Lymphadenopathy None / Cervical / Axillary / Inguinal / Multiple

Skin & hair Normal / Pellagra / Silky hair / Keloid

Jaundice / Pallor / Oedema / Clubbing / Cyanosis / Goitre

BCG scar / Smallpox jab scar

Oral candida / Oral KS

Other _____

CVS Normal / Abnormal _____

Signs of rheumatic heart disease / Signs of LVF / Signs of RVF

RS Normal / Abnormal _____

Wheeze / Fibrosis / Effusion / Consolidation

GI Normal / Abnormal _____

Surgical scars / Hepatomegaly / Splenomegaly / Palpable kidneys / Mass / Ascites

Peripheral neuropathy No / Yes

Other _____

Test results

HIV

Date of test __ __ / __ __ / __ __ (dd/mm/yy)

Negative / Positive CD4 count (if positive) __ __ __

Stool

Date of test __ __ / __ __ / __ __ (dd/mm/yy)

Result Negative / Asc. / Strong. / Enterobius / Taenia / Schisto. / Trich. / Hook.

Other _____

Treatment Albendazole / Mebendazole / Praziquantel / Other _____

Treatment started __ __ / __ __ / __ __ (dd/mm/yy)

Notes

Date ___ ___ / ___ ___ / ___ ___ **Pre / Post / Control** **ID** ___ ___ ___ ___

Confocal laser endomicroscopy	**Y / N**	**Settings**	**(Default)**
Pethidine (mg)	**0 / 25 / 50 / ____**	**Gamma** __ . __ __	**(1.74)**
Diazepam (mg)	**0 / 5 / 7.5 / ____**	**Bright** __ . __ __	**(0.47)**
Midazolam (mg)	**0 / 2.5 / 5 / ____**	**Laser** __ __ __	**(380)**
Buscopan (mg)	**0 / 10 / 20**	**Operator**	**PK / JLA**

Indication **(controls only)** Dysphagia | GORD | Dyspepsia/Abdo pain | Wt loss | Abn. radiology | Planned rpt |

Other _____

Oesophagus **Not seen / Normal / Stricture / Candida / Varices / Cancer / Ulcer / Other** _____

Passable / Impassable **(if relevant)** **Benign / Malignant** **(if relevant)**

Stomach **Not seen / Normal / Varices / Cancer / GU / Gastritis / Pyloric stenosis / Other** _____

Passable / Impassable **(if relevant)** **Benign / Malignant** **(if relevant)**

Duodenum **Not seen / Normal / Stricture / DU / Duodenitis / Other** _____

Passable / Impassable **(if relevant)**

iScan grade **Normal / Leaves / Ridges / Convolutions / Atrophic**

Micro grade **Normal / Leaves / Ridges / Convolutions / Atrophic**

Timings checklist

- ☐ Baseline blood ___ ___ : ___ ___
- ☐ Baseline urine ___ ___ : ___ ___
- ☐ Fluorescein ___ ___ : ___ ___
- ☐ Biopsies ___ ___ : ___ ___
- ☐ Solutions ___ ___ : ___ ___
- ☐ 3h blood ___ ___ : ___ ___
- ☐ 3h urine ___ ___ : ___ ___
- ☐ Digest started ___ ___ : ___ ___

Intubation depth **<D1/2 | D1/2 | D2 | D2/3 | D3 | >D3**

ADVERSE EVENTS / NOTES: PTO

Date ___ ___ / ___ ___ / ___ ___ Pre / Post / Control ID ___ ___ ___ ___

Anthropometry

Grip strength Y / N ____ ____ . ____ kg

BodPod Y / N

Bioelectrical impedance Y / N

NOTES

TROPGAN AMAZE study: Monthly follow-up case report form ID ___ ___ ___ ___

Date ___ ___ / ___ ___ / ___ ___ (dd/mm/yy) **Study week number** **4** **8** **12** **End**

Weight (end-of-study visit only) ___ ___ ___ . ___ *kg* *MUAC (end-of-study visit only)* ___ ___ . ___ *cm*

MM tabs remaining ____ ____ **AA packets remaining** ____ ____

How easy is it for you to remember to take both your medicines every day? Very easy | Quite easy | Quite difficult | Very difficult

How easy is it for you to take all your medicines? Very easy | Quite easy | Quite difficult | Very difficult

How often have you forgotten to take your medicines?

Most days | 2 or 3 times a week | About once a week | Less than once a week | Once or twice | Never

Describe any side effects _____

Describe any problems with taking the medicines _____

In the last month (since your last visit) have you had...

Diarrhoea (liquid stool at least 3 times a day) **No** | **Yes**

How long did it last? 3 days or less | More than three days

Was there blood? No | Yes

Time off your usual activities? No | Yes

Treatment given None | ORS | Abx (self-prescribed) | Abx (clinic/hospital) | Admitted | Other

Any other symptoms / illnesses **No** | **Yes**

Details _____

Treatment given _____

Medical attention None | St Lawrence or other clinic (not admitted) | Admitted

In the last month (since your last visit) have you taken...

Antibiotics / antimalarials **No** | **Yes**

Name _____ | Unknown

For how many days? 1 | 2-3 | Up to a week | More than a week

Prescriber Self | Clinic/hospital

Indication Fever | Cough | Diarrhoea | Malaria (no blood tests) | Malaria (confirmed with tests) | Other

Details _____

Pain killers **No | Paracetamol | Ibuprofen | Diclofenac | Indomethacin | Other_____ | Unknown**

For how many days? 1 | 2-3 | Up to a week | More than a week

Prescriber Self | Clinic/hospital

Have you taken any other medicines or remedies (apart from your regular medicines)? No | Yes

Details _____

AMAZE **Unplanned clinic attendance questionnaire** **ID __ __ __ __**

Date (dd/mm/yy) __ __ / __ __ / __ __

Current problem(s)

Clinical findings

Impression

Plan

Related to study?

Definitely / Probably / Possibly / Remote / Not

SAE?

No (0) / Yes (1)

Appendix 4. ImageJ morphometry macro.

```
//Morphometry of intestinal biopsy sections v1 (C) 2016 John Louis-
Auguste (j.l.auguste@qmul.ac.uk)

//.csv results file headings:
//Base length ('B') = 'Perim.' or 'Length'
//Villus area ('VA/VP') = 'Area'
//Villus perimeter ('VA/VP') = 'Perim.' [base length needs to be
subtracted from this value]
//Villus height ('VH/VW') = 'Width'
//Villus width ('VH/VW') = 'Height'
//Muscularis mucosae length ('MM') = 'Perim.' or 'Length'
//Crypt depth ('CD') = 'Perim.' or 'Length'
//All other values should be ignored / deleted

run("Open...");
//run("Enhance Contrast...", "saturated=5");
run("8-bit");
run("Set Scale...", "distance=1.1 known=1 pixel=1 unit=um global");
//Sets scale for 10X objective
run("Set Measurements...", "area perimeter bounding display
redirect=None decimal=3");
run("Colors...", "foreground=black background=white selection=red");
run("8-bit"); //Converts to 8-bit image for thresholding
rename(File.nameWithoutExtension);

//Select all villi to be measured as a group on duplicated 8-bit image
run("Select All");
run("Copy");
newImage(File.nameWithoutExtension, "8-bit white", 1680, 1056, 1);
run("Paste");
newImage(File.nameWithoutExtension, "8-bit white", 1680, 1056, 1);
run("Paste");
run("Select None");
setTool("polygon");
waitForUser("Draw round all villi to be measured (double click to
complete) then press OK. Draw across villus bases accurately!");
run("Make Inverse");
run("Cut"); //Leaves villi only

//Define function to rename last RoI and leave it selected
function roiRenameLast(name) {
last = roiManager("count")-1;
roiManager("select", last);
roiManager("Rename", name);
}

//Draw and measure all bases first
var n = 0
run("Select None");
while (getBoolean("Draw a villus base?") == 1)  {
      setTool("polyline");
      run("Line Width...", "line=5");
      waitForUser("Draw villus base (double click to start & complete)
then press OK");
      roiManager("Add");
      n++;
      roiRenameLast(" B"+n);
      roiManager("Measure");
      setForegroundColor(0, 0, 0);
      run("Fill", "slice");
}

//Delete all bases from ROI manager
selectWindow("ROI Manager");
run("Close");

//Threshold villi for automated measurements
run("Auto Threshold", "method=Default white");
```

```
setTool("wand");
waitForUser("Check thresholding by clicking on each villus in turn.
Correct defects etc. using PENCIL TOOL then press OK. [F1 or Edit-
>Options->Colors...->'Foreground': white=delete / black=draw]");

//VP, VA, VH, VW measurements for each villus
n=0;
while (getBoolean("Measure villus parameters?") == 1) {
      run("Select None");
      setTool("wand");
      waitForUser("VA/VP measurement: Click on a villus then press OK.
Ensure correct villus perimeter is outlined in red.");
      roiManager("Add");
      n++;
      roiRenameLast(" VA/VP"+n);
      roiManager("Measure");
      setTool("polyline");
      run("Line Width...", "line=500");
      waitForUser("VH/VW measurement (1/3): Draw villus long axis
(double click to start & complete) then press OK");
      run("Straighten...");
      setTool("wand");
      waitForUser("VH/VW measurement (2/3): Click on selected villus
then press OK. Ensure correct villus perimeter is outlined in red.");
      run("To Bounding Box");
      waitForUser("VH/VW measurement (3/3): Adjust bounding box if
required, then press OK");
      rename(File.nameWithoutExtension);
      roiManager("Add");
      roiRenameLast(" VH/VW"+n);
      roiManager("Measure");
      roiManager("Delete");
      run("Close"); //Closes bounding box image
      }

run("Close"); //Closes thresholded image

//Draw MM
roiManager("Show All"); //Shows all measured villi for assessing MM
setTool("polyLine");
run("Line Width...", "line=1");
waitForUser("Draw MM (double click to complete) then press OK");
roiManager("Add");
roiRenameLast(" MM");
roiManager("Measure");

//Draw crypts
n=0;
while (getBoolean("Draw crypt?") == 1)    {
      setTool("polyline");
      run("Line Width...", "line=1");
      waitForUser("Draw crypt (double click to start & complete) then
press OK");
      roiManager("Add");
      n++;
      roiRenameLast(" CD"+n);
      roiManager("Measure");
      }

//Save 8-bit image with overlays, save results as csv, tidy up
roiManager("Show All");
run("Line Width...", "line=1");
run("Flatten");
rename(File.nameWithoutExtension+"_overlay");
saveAs("tiff"); // Use saveAs(format, path) to save to specific
directory
saveAs("results"); // Use saveAs(format, path) to save to specific
directory
selectWindow("ROI Manager");
run("Close");
run("Close All");
runMacro("/Applications/Fiji.app/macros/AutoRun/Morpho.ijm");
```

Appendix 5. Incubation media for MTORC1 flow cytometry assay.

	Nutrient deprived	Nutrient rich
FBS (lot specific)	–	10%
Glucose	5.56mM	11.11mM
L-Glutamine	1mM	4mM
L-Glycine	–	0.13mM
L-Arginine	–	1.15mM
L-Asparagine	–	0.38mM
L-Aspartic acid	–	0.15mM
L-Cysteine	–	0.21mM
L-Glutamic acid	–	0.14mM
L-Histidine	–	0.10mM
L-Hydroxyproline	–	0.15mM
L-Isoleucine	–	0.38mM
L-Leucine	–	0.38mM
L-Lysine	–	0.22mM
L-Methionine	–	0.10mM
L-Phenylalanine	–	0.09mM
L-Proline	–	0.17mM
L-Serine	–	0.29mM
L-Threonine	–	0.17mM
L-Tryptophan	–	0.02mM
L-Tyrosine	–	0.11mM
L-Valine	–	0.17mM
Gentamicin	50µg/mL	50µg/mL
Penicillin G	100U/mL	100U/mL
Streptomycin	100mg/mL	100mg/mL

Table S1. In addition, RPMI in the nutrient rich solution contains a number of vitamins, and both solutions contained balanced inorganic chloride, phosphate, bicarbonate salt solutions (calcium, magnesium, potassium, sodium).

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